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19. ABSTRACT We have been engaged in a multimethod study of Rift Valley fever (RVF) virus in mosquitoes. During this year, we have carried out: (1) immunocytochemical and ultrastructural studies of the proventriculus of adult, female <u>Culex pipiens</u> infected with RVF virus. (2) immunocytochemical studies of the salivary glands and other tissues in <u>Anopheles</u> sp. which had been shown to be non-transmitters when intrathoracically (IT)-infected as adults, but transmitters when IT-infected as immatures. (3) work on the development of an immunogold procedure for <u>in situ</u> labelling of RVF virions in					
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Fever Virus In Mosquitoes

electron microscopic preparations. (4) work on the application of a cDNA probe for the in situ localization of RVF viral genome in serial paraffin sections of mosquitoes. (5) work on the identification of the mosquito cell surface receptor molecule for RVF virus. (12P)

Our major findings and successes have been as follows: (1) We have described in detail the histology and ultrastructure of the proventriculus of Cx pipiens. (2) We have found additional evidence, both immunocytochemical and ultrastructural, that RVF virus can disseminate via cells located at the midgut-foregut junction. (3) We found evidence that RVF virions are assembled in association with smooth membranes and have observed budding of RVF virions through the basal-lateral membrane of cardiac epithelial cells into the basal labyrinth. We have also observed budding of RVF virions through the plasma membrane into the "endocuticle" of the foregut intima. (4) We have made progress in the development of immunogold-labelling of RVF virions in ultrathin sections. (5) We have found evidence of a "salivary gland infection barrier" in Anopheles sp. This "barrier" is possibly time-dependent. (6) The cDNA probe has been used successfully for the in situ labelling of RVF viral genome; (7) We have made progress toward finding the mosquito cell surface receptor for RVF virus, particularly by developing a polyclonal antiserum to cell membrane extracts of Aedes albopictus C6/36 cells which significantly reduced RVF viral plaque formation on Vero cell monolayers. To facilitate isolation of this receptor, we are currently preparing monoclonal antibodies from this polyclonal serum in hopes of finding one or more antibody species which bind to the RVF viral receptor.



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STUDIES OF INFECTION AND DISSEMINATION OF RIFT VALLEY FEVER
VIRUS IN MOSQUITOES

ANNUAL AND FINAL REPORT

William S. Romoser

May 1990

Supported by

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official Department of the Army position unless so
designated by other authorized documents

SUMMARY

An final project summary is included at the end of this report and includes a description of objectives, methods, and major findings.

During this year, we have carried out the following studies: (1) Immunocytochemical and ultrastructural studies of the proventriculus of adult, female Culex pipiens infected with Rift Valley fever (RVF) virus. (2) Immunocytochemical studies of the salivary glands and other tissues in Anopheles sp. which had been shown to be non-transmitters when intrathoracically-infected as adults, but transmitters when intrathoracically-infected as immatures. (3) Worked on the development of an immunogold procedure for in situ labelling of RVF virions in electron microscopic preparations. (4) Worked on the application of a cDNA probe for the in situ localization of RVF viral genome in serial paraffin sections of mosquitoes. (5) Worked toward the identification and isolation of the mosquito cell surface receptor molecule for RVF virus.

Our major findings and successes this year are as follows: (1) We have described in detail the histology and ultrastructure of the epithelial layers and associated tissues in the proventriculus (cardia) of Culex pipiens. (2) We have provided additional evidence, immunocytochemical and ultrastructural, that RVF virus can disseminate via cells located at the midgut-foregut junction and have provided evidence for several possible routes of viral movement. (3) We have demonstrated that the proventriculus is an excellent tissue in which to study RVF viral morphogenesis since in IT-infected specimens, this structure, especially the intussuscepted foregut is, after an adequate incubation period, consistently infected. (4) We found evidence that RVF virions are assembled in association with smooth membranes and have observed budding of RVF virions through the basal-lateral membrane of cardiac epithelial cells into the basal labyrinth. We have also observed budding of RVF virions through the plasma membrane into the "endocuticle" of the foregut intima. (5) We have made progress in the development of immunogold-labelling of RVF virions in ultrathin sections. (6) We have found very promising evidence of a "salivary gland infection barrier" in Anopheles sp. This "barrier" is possibly time-dependent. (7) The cDNA probe has been used successfully for the in situ labelling of RVF viral genome; (8) We have made progress toward finding the mosquito cell surface receptor for RVF virus, particularly by developing a polyclonal antiserum to cell membrane extracts of Aedes albopictus C6/36 cells which

significantly reduced RVF viral plaque formation on Vero cell monolayers. We are currently preparing monoclonal antibodies from this polyclonal serum in hopes of finding one or more antibody species which bind to the RVF viral receptor. This will facilitate isolation and characterization of this receptor.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication no. (NIH) 78-23, Revised 1978).

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I. Introduction

A. Background

The literature pertinent to arboviruses and mosquitoes and Rift Valley fever virus is extensively reviewed in Hardy et al. (1983), Hardy (1988), Meegan & Bailey (1988), Turell (1988), and in the original research contract proposal.

Since our research involves the use of a cDNA probe for the in situ localization of RVF virus in mosquito sections and various biochemical and immunochemical procedures for identification of the mosquito cell surface receptor molecule, literature pertinent to these two lines of research is briefly discussed below.

In situ hybridization is a procedure whereby labelled nucleic acid probes are annealed to target cellular mRNA or DNA (Singer, Lawrence and Vellave, 1986). It is a powerful technique for identifying foreign nucleic acid in specific parts of tissues or cells and a technique that we hope will clarify basic mechanisms involved in vertical transmission of arboviruses. Its chief advantage over Northern or Southern blot hybridization is that spatial information about the distribution of target within a tissue may be obtained. Our objective is ultimately to determine whether or not gene expression occurs in reproductive tissues and cells of Aedes mcintoshi to hopefully gain insight into the capacity of Ae. mcintoshi to vertically transmit Rift Valley fever virus. This species has been implicated in RVF vertical transmission (Linthicum, Davies, Kairo and Bailey, 1985). Since we have been unable to colonize Ae. mcintoshi in the laboratory, testing for evidence of transovarial transmission can only be done with field-collected specimens held in the laboratory. This being the case, we have used Culex pipiens as a model mosquito in which to develop our cDNA probe.

Cell surface receptors in general are discussed in Limbird (1986), while Lonberg-Holm & Phillipson (1981) deal specifically with virus receptors. Several researchers have described various approaches to isolation of viral receptors. Proia (1979) developed a procedure to isolate receptor membrane components with a non-ionic detergent and further fractionate the extract with a lectin affinity column. Moldow (1977), concerned with the preservation of the biological activity of the membrane constituents, used a lithium dithiodisuccinate extraction procedure to solubilize avian oncornavirus receptor. Butters and Hughes (1974) showed that non-ionic detergents and subsequent gel filtration resulted in a receptor-rich extract. Mischak

(1988) described an extraction procedure for the minor group receptor from HeLa cells and a purification procedure using ion exchange and gel filtration and Western Blot assay using non-denaturing SDS gel electrophoresis. Kayanaraman (1978) described a centrifugation preparation of membrane fractions after osmotic swelling of cells and manual Dounce homogenization. Mapoles (1985) described a purification procedure extracting virus-receptor complex from cell lysates. Nemerow (1986) purified the Epstein-Barr virus-C3D receptor complex by immunoaffinity chromatography. Schneider (1982) described a one-step immunoaffinity matrix method of receptor purification to find the transferrin receptor. Marriott (1987) extracted polyomavirus receptor moieties from mouse kidney cells using a non-ionic detergent. Kata and Akamatsu (1984) characterized glycoproteins using gel chromatography and Con A Sepharose lectin chromatography.

Tomassini and Colonna (1986) described an osmotic disruption of HeLa cells and subsequent crude separation by centrifugation, followed by a detergent solubilization. A receptor monoclonal antibody affinity column was used to extract the receptor from the solubilized cell extract. Gel filtration fractionation of the solubilized membranes, affinity purification and subsequent RIA assay resulted in a possible receptor protein, which was used to generate rabbit polyclonal antiserum. Subsequent membrane binding and cell protection assays demonstrated that the protein was indeed the virus receptor.

B. Objectives

The overall objective of this research is to contribute to our understanding of the epidemiology of Rift Valley fever. More specifically, our goals are (1) to describe the dissemination, pathogenesis, and morphogenesis of RVF virus in vector competent and incompetent mosquitoes and (2) to identify the mosquito cell surface molecule (receptor) to which RVF virus binds prior to entering a cell.

Ultimately we hope to shed light on intrinsic factors which influence vector competence using RVF virus/mosquitoes as model systems.

An additional objective is to contribute to studies of the dissemination, tissue tropisms and morphogenesis of selected Nairoviruses and unclassified bunyaviruses in ticks.

C. Research Covered in This Report

1. Immunocytochemical & Ultrastructural Studies
 - a. Studies of RVF virus in the proventriculus of adult Culex pipiens
 - b. Anopheles salivary gland barrier
2. Progress in the Development of the Application of a cDNA Probes for the In Situ Localization of RVF Viral Genome in Mosquitoes.
3. Progress in the Isolation and Characterization of the Mosquito Cell Surface Receptor Molecule for RVF Virus.

II. Materials and Methods

A. Introductory Comments

We have applied several methods for localizing viral infection in mosquito tissues. Each method has inherent advantages and disadvantages. Therefore the best approach is to use more than one method (Hardy, et al., 1983). To detect infectious particles in dissected organs and tissues, we are using plaque assay on Vero cells. To detect viral antigen, we are using the avidin-biotin-peroxidase complex (ABC) immunocytochemical technique for light level microscopy (Faran et al., 1986) and are also applying immunocytochemical techniques at the electron microscope level. To detect viral genome, we are involved in the development of a protocol for the application of a peroxidase-labelled complementary DNA probe to paraffin sections of whole mosquitoes. To detect whole virions and nucleocapsids, we are using standard transmission electron microscopy.

B. Plaque Assay

During this year we have used plaque assay primarily to determine the viral "doses" ingested by or injected into mosquitoes used in a given experiment. We have also used plaque assay to determine the dissemination status of a given mosquito.

A brief description of the plaque assay technique follows. Whole mosquitoes and mosquito parts are triturated in 1 ml of mosquito diluent (10% calf serum in Medium 199 with Hank's Salt and antibiotics) and tested for infectious

particles by plaque assay on 2- to 4-day-old Vero cell monolayers (Gargan et al., 1983). The mean amount of virus ingested by a sample of mosquitoes taken immediately following each infectious blood meal represents the viral "dose" for a given experiment. In order to determine whether or not virus is present in the body cavity (hemocoel), i.e. whether or not virus has disseminated from the midgut, legs are dissected and assayed.

C. ABC

Faran, et al. (1986) adapted the very sensitive ABC immunocytochemical technique (Hsu, et al., 1981) for use with serial paraffin sections of formaldehyde fixed, whole mosquitoes. The technique is based on the use of primary antibody directed against viral antigen, followed by biotinylated secondary antibody directed against immunoglobulin from the vertebrate in which the primary antibody was formed. Finally, a complex of avidin and biotinylated peroxidase is applied. This complex binds with the biotinylated secondary antibody due to the great affinity between biotin and avidin. The location of the primary antibody/secondary antibody/ABC complex is then rendered visible by the addition of diaminobenzidine tetrahydrochloride (DAB), the oxidative polymerization of which is catalyzed by peroxidase. The DAB polymer appears as a rusty brown precipitate. In our studies the "primary antibody" is actually a blend of monoclonal antibodies directed against RVF virus nucleocapsid protein and two envelope glycoproteins or a monoclonal antibody directed against RVF virus nucleocapsid protein. These antibodies are provided by Cdr. James Meegan and Dr. Jonathan Smith at USAMRIID.

D. Electron Microscopy

To prepare mosquito tissues for electron microscopy, tissues are (1) fixed in Karnovsky's solution (1.5% glutaraldehyde, 2.0 % formaldehyde in 0.1M phosphate buffer and 0.15M sucrose) for 1 1/2 to 2 hrs. at 0-4°C; (2) placed in buffered sucrose (15 min. to 48 hours); (3) post-fixed in 1.0% buffered osmium at 0-4°C for 1 hr.; (4) dehydrated by passing through an ethyl alcohol series; (5) placed in propylene oxide for 20 min., then 1:1 volumes of propylene oxide and resin (Epon 812; Araldite 502; DDSA; DMP-30) for 1 hr., the 1.0 ml of resin was added; (6) 3-24 hours later, tissues are embedded in aluminum foil pans and placed in an oven at 60°C for 48 hrs. Blocks of embedded tissues are cut on an ultramicrotome using a diamond knife, mounted on copper grids, and stained with uranyl acetate and lead citrate. Specimen grids are studied and electron

micrographs taken with a Zeiss 110 transmission electron microscope.

E. Immuno-electron microscopy Using Colloidal Gold

We have continued to try various approaches to developing the application of a post-embedding immunogold technique, using, in place of immunogold-protein A (last year), mouse monoclonal antibodies directed against RVF viral antigens along with a colloidal gold-labelled secondary antibody (anti-mouse).

F. Specific Investigations Involving Plaque Assay, the ABC Technique and Electron Microscopy

1. Studies of RVF virus in the proventriculus of adult Culex pipiens

Our objectives in this study have been as follows:

a. To elucidate the histology and ultrastructure of the proventriculus (cardia) of adult female Culex pipiens.

b. To continue to test the hypothesis that Rift Valley fever virus can disseminate from the midgut lumen via cells at the foregut-midgut junction by applying:

(1) the ABC immunocytochemical technique and optical microscopy to study the proventriculus in intrathoracically (IT) infected mosquitoes.

A series of IT-infected mosquitoes was examined on days 2, 4, and 6 following infection to determine the sequence of infection of the proventriculus by virus from the hemocoel. Our operating assumption was that if RVF virus readily disseminates from the midgut by cell to cell spread in cells in the region of the midgut-foregut junction, then the reverse may also be true. That is, virus from the hemocoel should, by cell to cell spread, sequentially infect IF cells, then ACE cells, and finally CE cells, the overall region acting as a "viral conduit."

(2) electron microscopy to check for the presence of RVF virions in the IF or adjacent esophageal epithelium in specimens with non-disseminated infections.

c. To identify, on the basis of various tissues in which putative RVF virions have been observed, possible routes of egress associated with cells at the midgut-foregut junction.

2. *Anopheles* salivary gland barrier

a. Turell (1988) reported that the ability of *Anopheles albimanus* to transmit RVF virus was determined by the developmental stage during which they were IT- infected. All adult mosquitoes which had been infected as larvae transmitted virus, but only a very small fraction of specimens IT-infected as adults and incubated for an equivalent period of time transmitted virus. Since "...Viral titers were similar for all groups tested...", he suggested the possibility that "...differences in transmission rates may have been due to site-specific (i.e., salivary gland) replication, rather than a generalized increase in viral replication in mosquitoes inoculated at an earlier age." He further suggested that "Perhaps inoculation of larvae allowed virus to enter cells in the primordial tissue that was destined to become adult salivary glands, which might otherwise become refractory to infection during metamorphosis to the adult stage."

b. On the basis of the above and additional data, Dr. Turell and personnel in our laboratory began a collaborative study to see if in fact the differences in transmission involved differences in the infection of the salivary glands.

M. Turell IT-infected 10 fourth instar larvae, 18 pupae of different ages post-pupation, and 20 adults with RVF virus. All specimens were incubated for equivalent lengths of time, time enough for all specimens to reach the adult stage and become ready to blood feed. All specimens were tested for their ability to transmit virus to a hamster. In addition, 10 uninfected, negative control adult mosquitoes were given blood meals. Following the transmission tests, all specimens were coded and given to W. Romoser who fixed them and prepared serial paraffin sections according to the methods outlined in Faran et al., 198 and described earlier in this report. The ABC procedure was applied to all slides. Neither the infection state nor the time of infection was known to the persons (Lerdthusnee & Romoser) who would be interpreting the immunocytochemically stained slides.

G. Development and Application of the cDNA Probe

Rift Valley fever complementary DNA (cDNA) cloned into the plasmid vector sp76-6 was obtained from M.S. Collett. The 3.3 kb cDNA probe contains the Rift Valley fever virus M segment encoding the viral glycoproteins G1 and G2, the principal determinants of virulence and virus transmission.

The recombinant plasmid was propagated in Escherichia coli strain DH5 F¹ and purified by standard procedures (Hanahan, 1983, J. Mol. Biol., 166:557-580). Linearized plasmid was labelled with digoxigenin-11-dUTP by random hexanucleotide primary using the Genius Non-radioactive DNA labelling and Detection Kit (Boehringer Mannheim) following the protocol supplied by the manufacturer.

Aedes mcintoshi, Aedes circumluteolus, and Culex pipiens mosquitoes were intrathoracically inoculated with Rift Valley fever virus and held for 7 days at 28°C. Mosquitoes were fixed in 4.0% paraformaldehyde for 4 hr at RT and embedded in paraffin blocks using standard histological procedures. Histological sections were cut into 5-7 um thickness and mounted on slides coated with 20% Elmer's glue. Tissue sections were deparaffinized and rehydrated by sequential immersion into xyless (2 x 3 min), absolute ethanol, 95% ethanol, 90% ethanol, 70% ethanol, distilled water (2 min each), and 2X SSC (10 min). the tissue sections were then ready for DNA hybridization.

Both the pre-hybridization and hybridization mixture contains a final concentration of 4X SCC, 50% formamide, 1X Denhardt's solution, 5% dextran sulfate, 0.5% mg/ml salmon sperm DNA, and 0.25 mg/ml tRNA. Sections were prehybridized for 1 hr at RT. The RVF cDNA probe labelled with digoxigenin-11-dUTP was diluted (1:250, 1:500, 1:700 and 1:1000) in prehybridization buffer denatured at +100°C for 10 min, and quick-cooled in an ice bath. Approximately 200 ul of hybridization solution was utilized per slide. Hybridization was carried out at 37°C in a humid incubator for 16 hours.

Following hybridization, the sections were washed in 2 changes of 2X SSC (30 min, 10 min), 1X SSC (10 min) and 0.5X SSC (10 min) at RT. For immunological detection of hybrids slides were washed in buffer 1 (100 mM Tris HCL, pH 7.5 150 mM NaCl) containing 2% normal sheep sera to block non-specific binding of the sheep anti-digoxigenin antibody for 30 min at RT. Sections were incubated for 2 hr with

anti-digoxigenin antibody conjugated to alkaline phosphatase. One μ l of the antibody conjugate was diluted with 500 μ l of buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100. Slides were washed two times (15 min each) with buffer 1 and then were rinsed for 2 min in buffer 3 (100 mM TrisHCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$). The color detection solution was made by mixing 4.5 μ l of a 70% stock solution [V/V] of nitroblue tetrazolium salt in dimethylformamide with 3.5 μ l of 50 mg/ml stock of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in dimethylformamide and 2.4 μ l of levamisole (to inhibit endogenous alkaline phosphatase activity) in a final volume of 1 ml in buffer 3. Approximately 200 μ l of this solution was used per slide. The color was allowed to develop overnight at RT. The color reaction was stopped in buffer 4 (100 mM Tris, pH 7.5, 150 mM NaCl, EDTA). Slides were rinsed in 2 changes of distilled water (2 min each). Slides were then dehydrated by sequential immersion into 70% ethanol, 90% ethanol, 95% ethanol, and absolute ethanol (2 min each), and placed in 2 changes of xyless (3 min, 2 min). Slides were then mounted in Permount, allowed to dry and examined microscopically.

For paraffin-embedded sections, mosquitoes are fixed in 4.0 % paraformaldehyde overnight at 4°C. The mosquitoes are embedded in paraffin by standard histological procedure. Serial sections are cut (7-10 μ m) and floated onto 20% Elmer's Glue treated slides. Slides are left on a 42°C slide warmer for 2 hours, allowing the sections to adhere to the slides.

Sections are deparaffinized with xyless and hydrated through a graded series of alcohol solutions to water. Sections are washed with 2XSSC and then prehybridized in a buffer that contains inhibitors to reduce background by competitively inhibiting non-specific binding of nucleic acids.

A RVF virus cDNA probe labelled with digoxigenin dUTP is hybridized to cellular RNA. RNA-DNA hybrids are detected by enzyme-linked immunoassay using an anti-digoxigenin alkaline phosphatase conjugate and enzyme catalyzed color producing substrate.

H. Techniques applied in the search for the receptor

1. Introduction

We are using two somewhat different approaches in our attempt to identify, isolate and characterize the mosquito cell surface receptor molecule to which RVF virus attaches prior to infection of the host cell. These two approaches are described below.

One approach involves the application of various molecular extraction and separation techniques to mosquito cells and/or tissues with the subsequent use of virus preparations as "probes" and virus detection assay(s) to identify fractions to which virus binding occurs. When one or more "binding" fractions is identified, they will be used as antigens in the production of monoclonal antibodies. These antibodies to putative receptor epitopes would be expected to reduce or inhibit infection of mosquito and/or vertebrate cells in culture or within whole mosquitoes.

The other approach begins with the development of monoclonal antibodies to mosquito cell surface molecules which are then screened on the basis of binding to mosquito cell surfaces (both cultured cells and cells/tissues dissected from whole mosquitoes) and on the basis of reduction or inhibition in viral plaque (infectivity) assays using mosquito and vertebrate cell lines. The MAbs which bind and which cause a degree of inhibition in infectivity assays will then be used in an immunoaffinity column in an attempt to isolate the mosquito cell surface receptor molecule(s) from a mosquito cell membrane extract.

In addition to the above approaches, we are currently considering other methods, possibly involving the use of radioisotopes and immunoprecipitation. Initially, we tried to isolate viral protein from the virus preparations described below, i.e. formalin-killed vaccine virus and RVF virus HA antigen preparation, in order to use the viral proteins as ligands in affinity chromatography to isolate putative receptor molecules from mosquito cell membrane extracts. However, our attempts to purify viral protein from these preparations, and especially to remove the albumin fraction have been unsuccessful. Given the potential for success using other approaches, we decided to abandon use of the virus preparations as receptor-capturing ligands.

2. Mosquito Cell Preparations

a. We have three potential sources of mosquito receptor molecules available to us: (1) Aedes albopictus cells (C6/36) in culture; (2) homogenized whole mosquitoes; and (3) specific tissues and organs which can be dissected from whole mosquitoes. To date, we have employed the preparations described in B. & C. of this section.

b. Extract from Whole Mosquitoes: Adult Culex pipiens (El Gabal strain) were frozen and vortexed to sever appendages and then sifted through stretched nylon hose. The mosquitoes were mechanically disrupted in a 10 mM Tris-HCl extraction buffer consisting of 10 mM CHAPSO, 2 mM pepstatin, 2 mM PMSF, 100U/ml aprotinin and 0.2% NaN₃. The homogenates were centrifuged at 10,000 rpm for 30 min and the supernatant collected and dialyzed in bicarbonate buffer at pH 9.0.

c. Extract from Cultured Mosquito Cells (Aedes albopictus C6/36): Protein extraction from cultured cells grown in L-15 media supplemented with 10% FBS were performed in the same buffer as above, but the cells were vortexed gently and incubated at 4°C for 1 hr prior to centrifugation.

d. In assays in which whole Aedes albopictus cells were used, the cells were grown directly in 96 well plates which were then washed, fixed in 0.25% glutaraldehyde/PBS for 15 min., washed again and then used in the microtiter assay described below.

3. Approach #1--Extraction of putative receptor and use of virus probe

a. Four virus preparations have been potentially available to us: (1) live Rift Valley fever virus; (2) cobalt irradiated live RVF virus; (3) a mouse liver extract of the HA antigen of RVF virus, BPL inactivated; and (4) a formalin-treated virus vaccine preparation, prepared in monkey lung cell culture. Since use of live RVF virus would require that all work be done in the P-3+ containment facility at USAMRIID and since no cobalt irradiated RVF virus was immediately available and although feasible to prepare would require extensive safety testing before release from Fort Detrick, we opted to begin our research using the HA antigen preparation and the RVF vaccine preparation.

b. In order to justify the use the RVF virus HA antigen and RVF formalin-treated vaccine preparations, we first had to determine whether or not these preparations demonstrated specific binding to the surfaces of mosquito cells. If not we would be forced to use a different virus preparation. In order to determine the presence of such specific binding, we needed to develop one or more assays to detect the virus preparations.

c. Three assays, two quantitative and one qualitative, for detecting the virus preparations were developed:

(1) ELISA--Polystyrene plates were used as a solid phase for binding of solubilized albopictus cell extracts or whole mosquito body extracts. Sterile polypropylene plates were used for fixed, cultured albopictus cells.

In assays employing solubilized targets, the wells of the polystyrene plates were coated with saturating amounts of extract material (50 μ l of a 1 mg/ml protein solution as determined by the Lowery method) and incubated at 4°C for 20 hours. Non-specific binding was blocked by the addition of either 1 % BSA in PBS-0.05% Tween-20 or 5% Carnation low fat milk in the PBS-Tween buffer with 3% normal sheep serum for 2 hours. Subsequently, an appropriate dilution of the formalin-killed RVF virus vaccine preparation in 1% BSA/PBS/Tween-20 was used as the primary probe and incubated for 4 hours at RT. The plates were then washed 6 times with PBS/Tween-20 by an automated Dynatech plate washer. To detect the presence of virus which has remained after the washing steps (i.e. virus "bound" to putative receptor molecules), a mixture of monoclonal antibodies directed against viral surface glycoproteins (G1 & G2) and the nucleocapsid, N, protein was applied at a 1:1000 dilution in 1% BSA/PBS/Tween-20/3% NSS and incubated for 2 hrs. The plates were then washed again followed by another 2 hrs. of incubation with an HRP conjugated sheep anti-mouse immunoglobulin.

(2) Biotinylated Virus Assay--RVF virus vaccine (formalin-treated) was biotinylated using a succinimide ester of sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-L-biotin) according to the method of Gretch et al. (1987). The biotinylated virus preparation was then used as the primary probe, i.e. the probe which binds to putative cell surface receptor molecules. The coating of antigen and blocking (excluding the use of milk and NSS) were performed as in the ELISA described above. Following the blocking step, 50 μ l of biotinylated-RVF virus (BRVfV)

at a final concentration of 1 ug/ml was applied and incubated for 4 hrs at RT. The plates were then washed 6 times and a 1:1000 dilution of streptavidin conjugated with HRP in PBS/Tween-20 was applied. The plates were further incubated for 45 min at 37°C, washed, developed with OPD and the optical density measured at a dual wavelength of 490 nm and 405 nm.

(3) Immunofluorescence Assay--One-half ml of suspended C6/36 cells was placed on a sterile round cover slip that was placed in the bottom of a 24-well culture plate. The cells were grown at 28°C for 6 days. The plate was centrifuged at 1000 rpm for 10 min; the supernatant was removed. One ml of cold 0.25% glutaraldehyde in PBS- 0.5% Tween 20 (PBS-T 20) was aliquoted onto the cover slips and incubated 15 min in the refrigerator. The wells were washed once with PBS-T 20. The cells were blocked 30 minutes with 2% normal sheep serum in PBS-T 20. After blotting the plates dry, viral antigen was added and incubated 90 min. The wells were washed 3 times with PBS-T20 and goat anti-mouse fluorescein-conjugated (1:100) was added and incubated 90 min. The wells were washed 3 times with PBS-T 20. The cover slips were mounted on clean slides with mounting media. The cells were examined with an epifluorescence microscope.

4. Approach #2--Development of Monoclonal Antibodies & Immuno- affinity Column

a. Four adult Balb/c mice were injected IP with washed, unfixed Aedes albopictus (C6/36) cells at 2 week intervals for 2 months. Serum from these animals was assayed for putative anti-receptor antibody by plaque inhibition assay using Vero cells. Serum from mice which showed signs of reduction of plaque formation will be used for the production of monoclonal antibodies directed against mosquito cell surface molecules (Kohler & Milstein, 1975).

b. Plaque inhibition assay (This experiment was carried out by Ralph Tammariello at U.S.A.M.R.I.I.D.):

In order to determine if any of the sera from these mice (designated MS, RE, LE, & NO E) contained antibodies that interfered with RVF virus infection of Vero cells (presumably by blocking access to viral receptor molecules on the cell surface membranes), the following experiment was carried out twice:

Reagents: RVF virus ZH-501 18 Nov. 86
Vero 76 6-well plates
Diluent - HBSS with HEPES, 2 % FBS, 50 ug/ml
gentamycin

- (1) Prepared 1:50 dilution of each mouse serum in diluent.
- (2) Placed 0.1 ml of #1 / well.
- (3) Incubated plates at 35°C for 1 hr.
- (4) Added 0.1 ml/well of a 1:2 dilution of 10^{-5} RVF virus.
- (5) Incubated plates at 35°C for 1 hr.
- (6) Overlayed each well with agar, 2.5 ml/well

0.5% agarose SeaKem
10% AFBS
50 ug/ml gentamycin
100 units pen
100ug/ml strep
(in 2X EBME with HEPES)

- (7) Incubated for 3 days at 35°C; 5% CO₂ in air.
- (8) Stained with 1 % neutral red (1:300)
- (9) Incubated overnight at 35°C.
- (10) Counted plaques.

III. Results and Discussion

A. Immunocytochemical & Ultrastructural Studies

1. RVF in the Proventriculus of Adult Culex pipiens

a. Histology and Ultrastructure of the Proventriculus

The proventriculus, as indicated by the boxed region in Fig. 1, is located in the anterior part of the mosquito thorax and is a complex organ in which the foregut ends and the midgut begins.

The proventriculus forms as an intussusception of both foregut and midgut epithelia (Fig. 1 & 2). The outer layer is midgut tissue, the cardiac epithelium. Inner layers consist of esophageal epithelium which enters the cavity formed by the cardiac epithelium and turns ectad (outward), forming the reflected (or recurrent) wall (Fig. 3, RW). The foregut intima ends at the base of the reflected wall of the esophagus.

The cardiac epithelium (Fig. 4) can be divided into two regions, anterior (ACE) and posterior (CE). Both regions of the cardiac epithelium have distinct microvilli (Mv) which project into the lumen. The microvilli of the ACE and CE are nearly opposite one another in places. Small regenerative cells (RC) can be seen at the bases of the ACE and CE cells. Interposed between the microvillate cardiac epithelial cells and the terminal cells of the intussuscepted foregut are cells which are lined on the entad (inward) side with cuticular intima from the larval foregut, i.e. the larval intima remnant. On the ectad side, these cells form junctions with the cells of the ACE. The larval intimal remnant represents the posteriormost section of the larval foregut intima (Fig. 5 & 6). During the pupal period this intimal remnant resembles a "windsock" and is directed anteriorly into the lumen of the developing foregut. Prior to the exit of the adult mosquito from the exuvia, this remnant changes direction, becoming directed posteriorly into the lumen of the midgut (Walker & Romoser, 1987). This "change in direction" has been interpreted as an indication of movement of fluid from the foregut into the midgut. Following adult emergence, the larval intimal remnant breaks away and only a short length

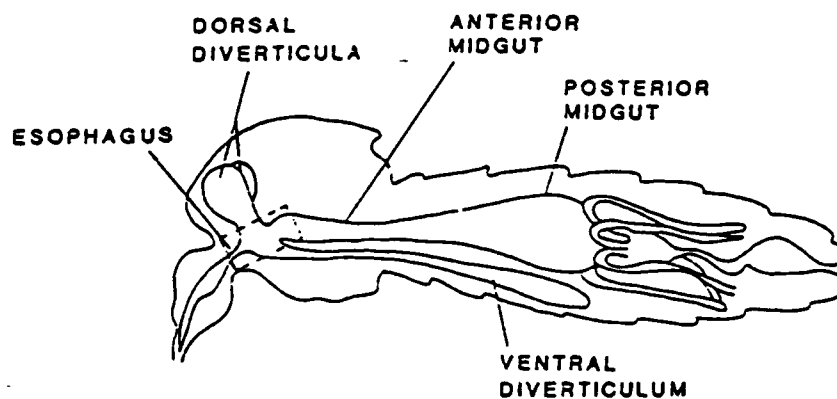


Fig. 1. Mosquito alimentary canal.

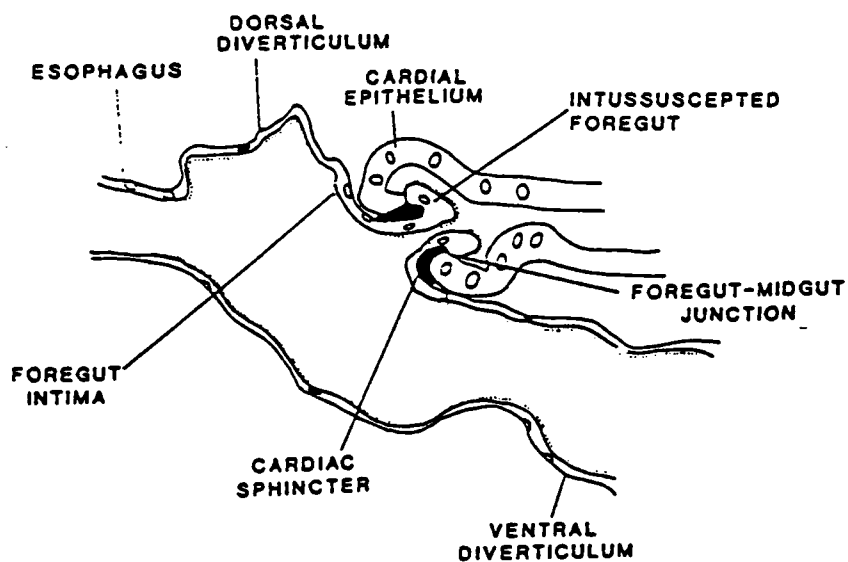


Fig. 2. Sagittal section in region of foregut/midgut junction (indicated by boxed area in Fig. 1).

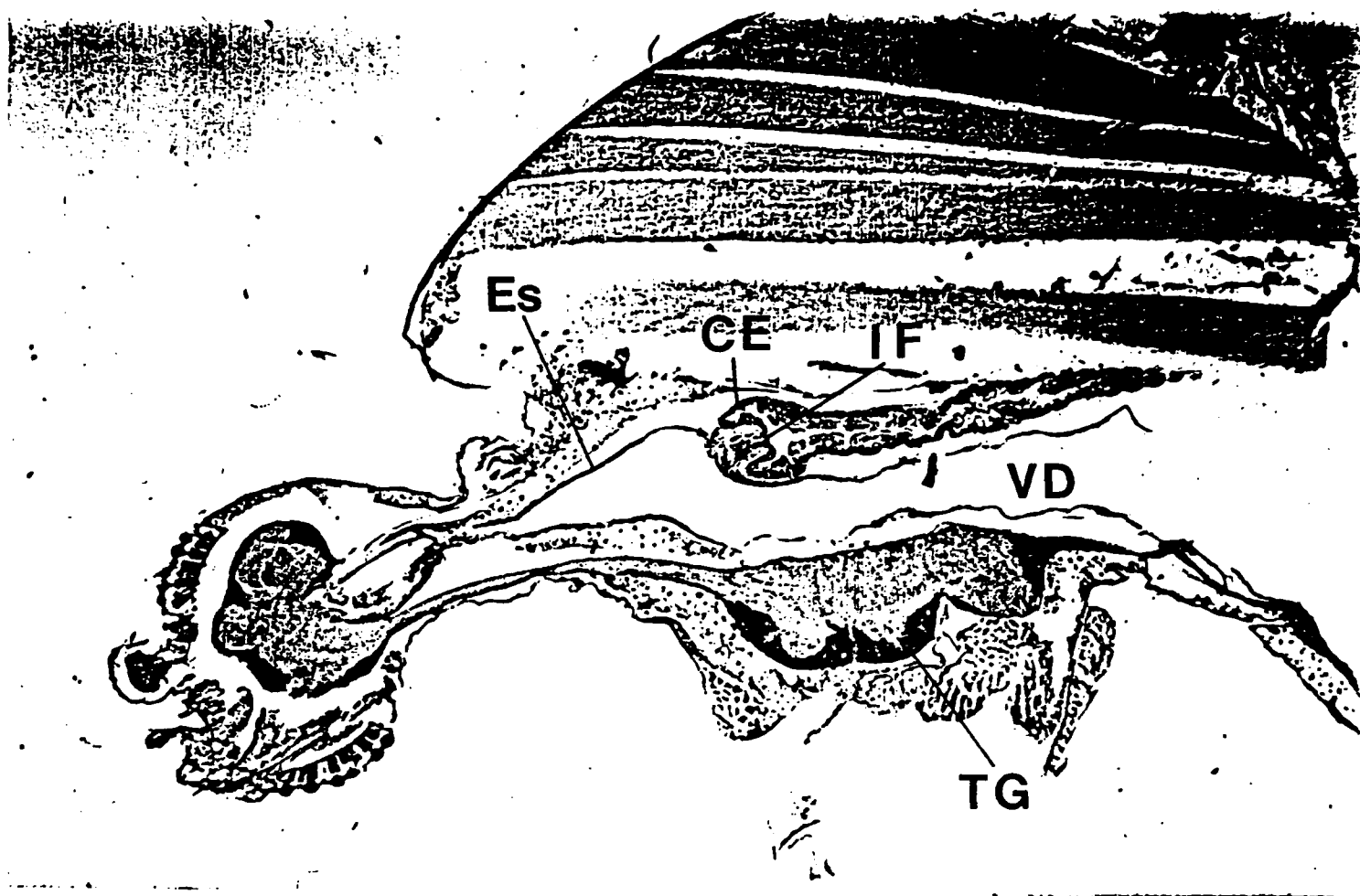


Fig. 3. Light photomicrograph of a sagittal section of the head and thorax of Culex pipiens. CE, cardiac epithelium; Es, esophagous; IF, intussuscepted foregut; TG, thoracic ganglia; VD, ventral diverticulum.

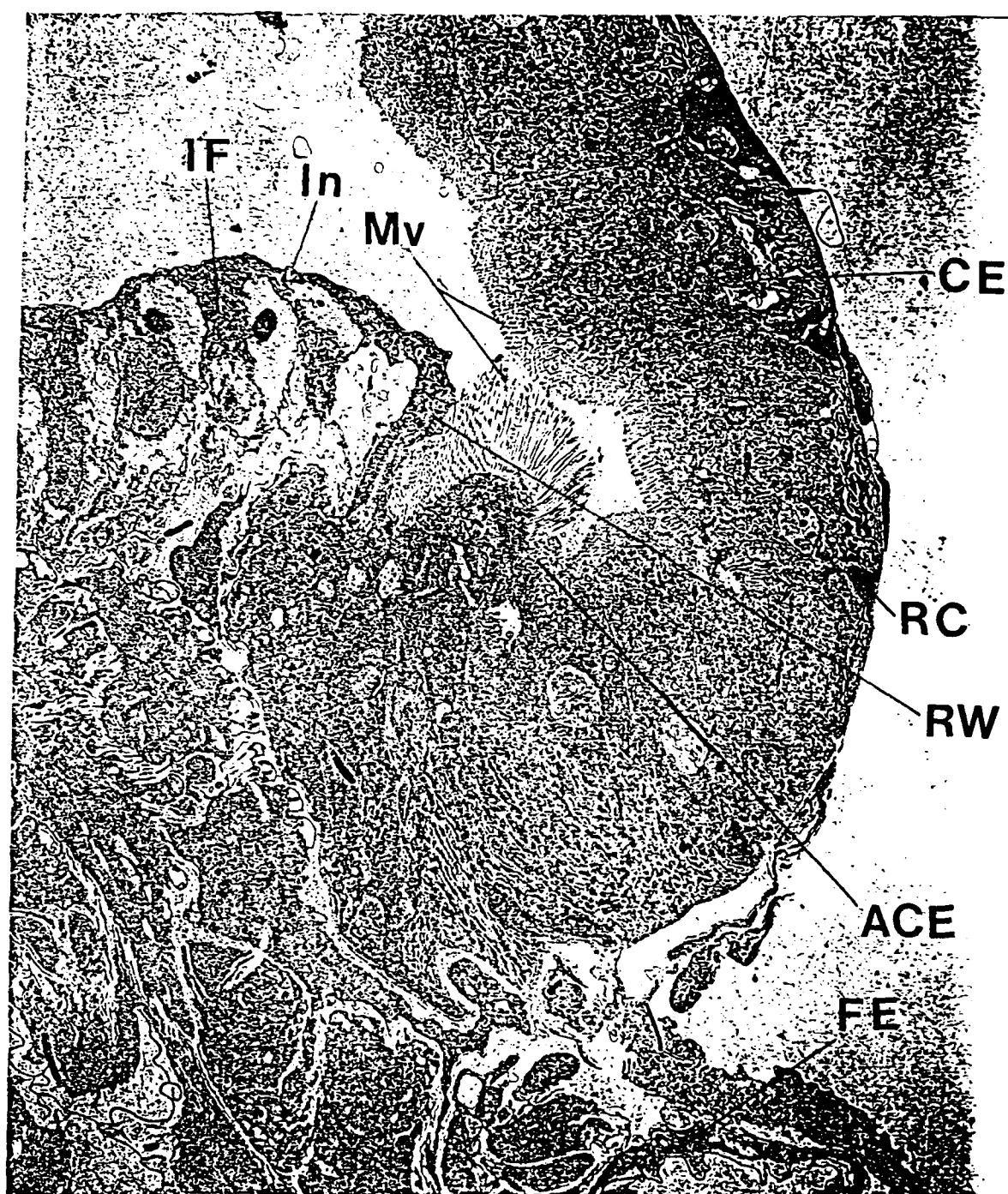


Fig. 4. Electron photomicrograph of sagittal section of portion of proventriculus of Culex pipiens. ACE, anterior cardiac epithelium; CE, cardiac epithelium; FE, foregut epithelium; IF, intussuscepted foregut; In, intima; Mv, microvilli; RC, regenerative cell; RW, reflected foregut wall.

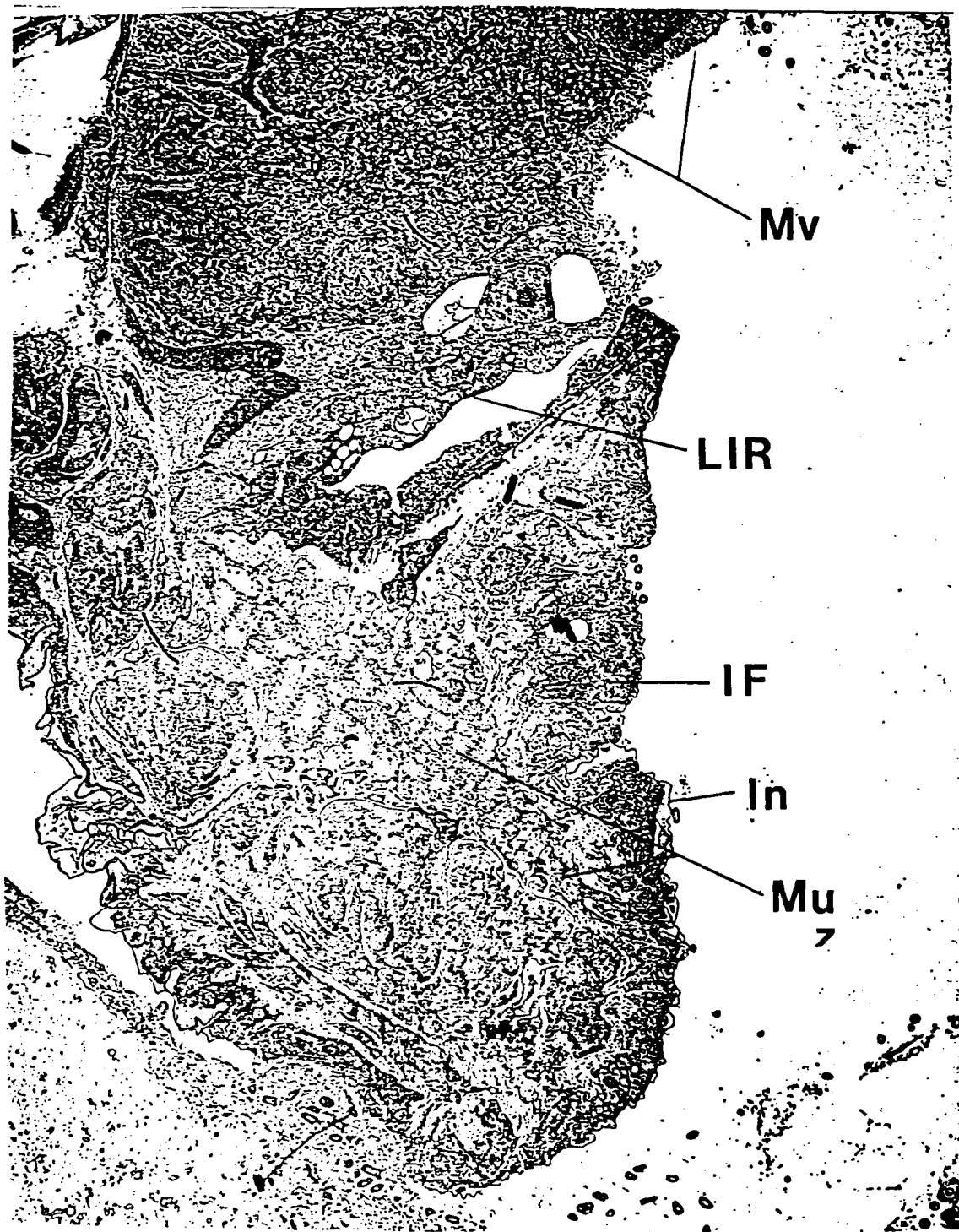


Fig. 5. Electron micrograph of sagittal section of proventriculus of Culex pipiens. IF, intussuscepted foregut; In, foregut intima; LIR, larval intimal remnant; Mu, muscle; Mi, microvilli.

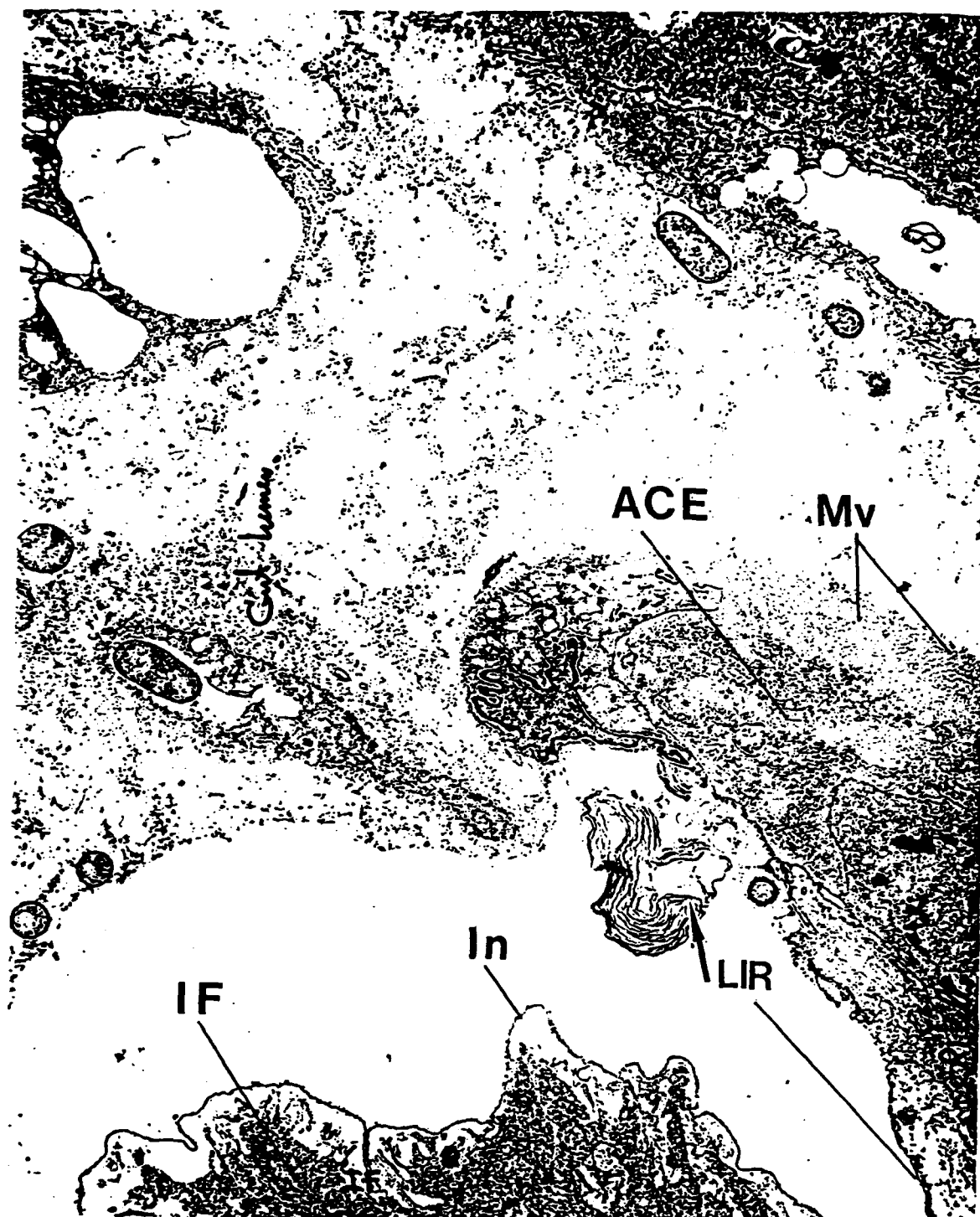


Fig. 6. Electron micrograph of portion of proventriculus of Culex pipiens showing multilayered piece of larval intimal remnant (LIR) which will probably slough into the lumen and a much thinner part of the larval intimal remnant (LIR) which represents the terminal part of the larval foregut intima. ACE, anterior cardiac epithelium; IF, intussuscepted foregut; In, foregut intima; Mv, microvilli of ACE.

of intima which represents the posterior termination of the larval intima remains attached as described above.

The foregut epithelium (Fig. 4, FE) enters the space surrounded by cardial epithelium and reverses on itself forming the intussuscepted foregut (IF) and reflected wall of the intussuscepted foregut (RW). The RW is continuous with the entad bases of the cells to which the larval intimal remnant is attached. For most of its length, the foregut intima appears to consist of an electron dense "epicuticle" and an electron lucent "endocuticle". As this intima approaches the foregut-midgut junction, the "endocuticle" tapers and finally, only "epicuticle" is apparent.

Three luminal regions are identifiable as the result of the intussusception of the foregut and the midgut: (1) the lumen of the esophagus; (2) the lumen between the reflected esophageal wall and the ACE/CE; and (3) the lumen between the esophageal epithelium which enters the proventriculus and the reflected esophageal epithelium, i.e. the hilus as referred to by King (1988).

Both extrinsic and intrinsic muscle fibers are evident in association with the proventriculus.

We have observed extrinsic muscles attached to the proventriculus in Culex pipiens and we assume they play a role in maintaining the position of the proventriculus in the thorax, that is act as suspensory muscles. Similar extrinsic muscles in Aedes triseriatus are traceable to the integument (Romoser & Venard, 1966).

With regard to intrinsic muscle fibers, both circular and longitudinal fibers can be found between the walls of the esophageal epithelium in the proventriculus (Fig. 7 & 8).

The circular muscles no doubt act collectively as a sphincter. Perhaps the circular and longitudinal muscle fibers act antagonistically, the longitudinals effecting opening of the inner lumen of the proventriculus and the circular fibers, closing of the proventriculus. It is also possible that the longitudinal fibers aid the circular fibers in closing the lumen by contracting the esophageal epithelium in a longitudinal direction.

Given the overall structure of the proventriculus, one gets the impression that the IF is

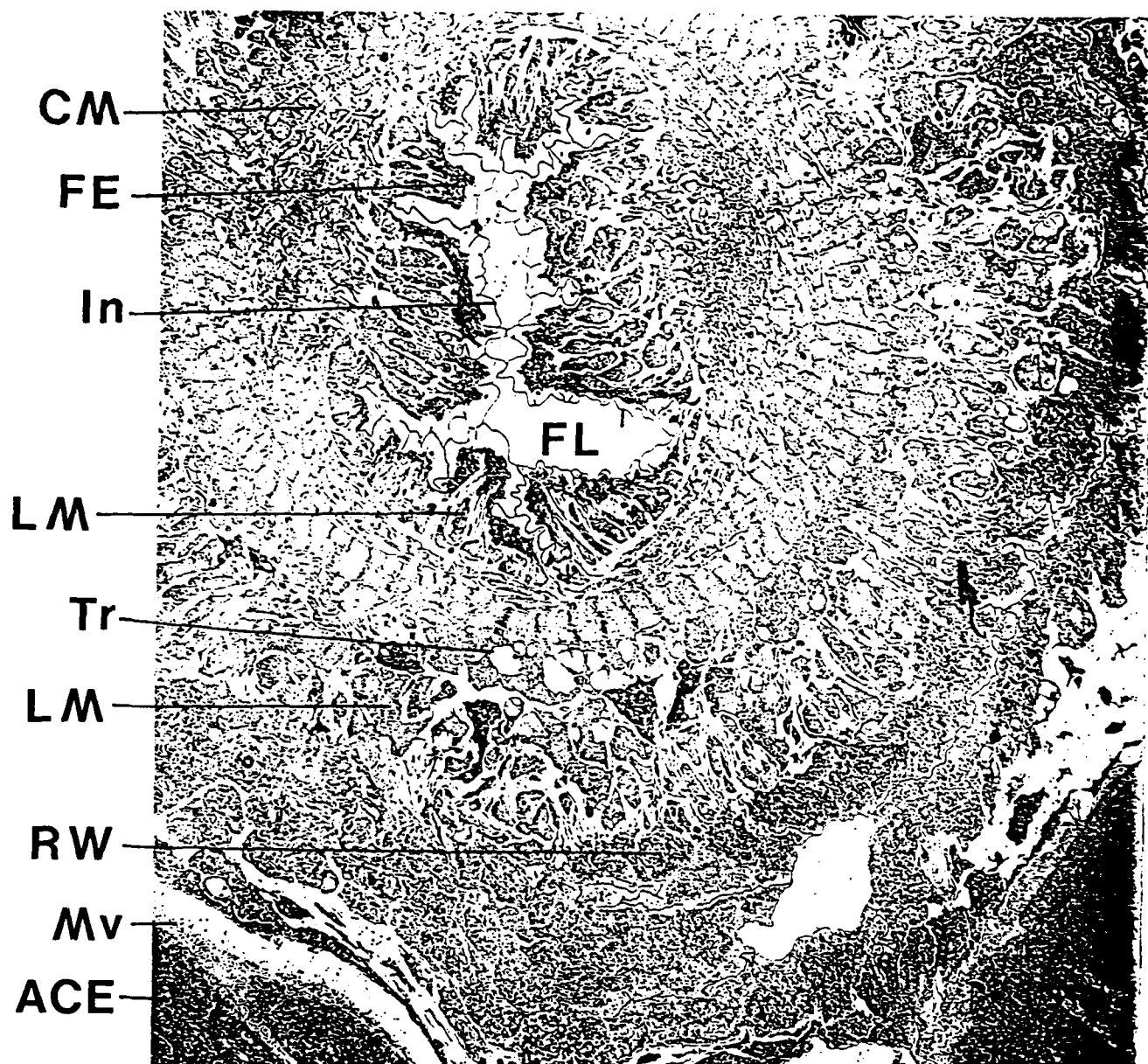


Fig. 7. Electron micrograph of a cross-section of proventriculus in Culex pipiens. ACE, anterior cardinal epithelium; CM, circular muscle; FE, foregut epithelium; FL, foregut lumen; In, foregut intima; LM, longitudinal muscle; Mv, microvilli; RW, reflected foregut epithelium; Tr, trachea.

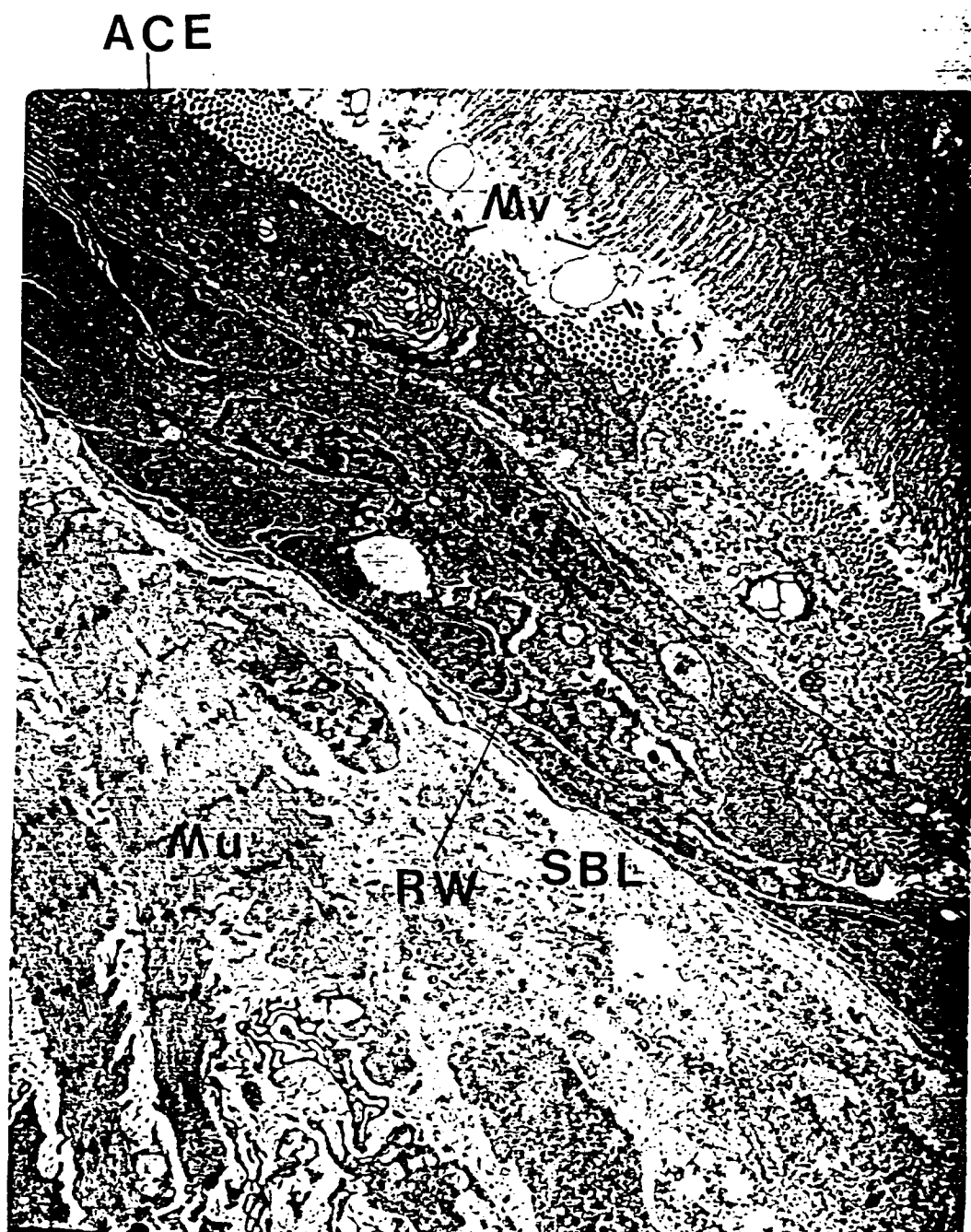


Fig. 8. Electron micrograph of section of proventriculus of Culex pipiens. ACE, anterior caridal epithelium; Mu, muscle; Mv, microvilli; RW, reflected foregut wall; SBL, "spongy" basal lamina.

capable of moving, at to some extent, in and out of the cavity formed by the cardial epithelium.

The midgut basal lamina enters the proventriculus as the cardial epithelial cells turn inward, and appears as a distinct, thin, moderately electron dense line. Within the proventriculus, just beyond the foregut-midgut junction, the midgut basal lamina merges into the loose, "spongy" layer or matrix between the layers of esophageal epithelium (Fig. 8).

The proventriculus is apparently well-supplied with tracheae and tracheoles which are particularly evident interspersed with the muscle fibers between the esophageal epithelium and the reflected esophageal epithelium.

We have not as yet studied the innervation of the proventriculus. However, other studies have shown that this structure receives innervation from the ventricular ganglia, one of which is located on either side of the proventriculus (Burgess & Rempel, 1966) and which, incidentally commonly becomes infected with RFV virus.

We have seen, within the proventriculus, what we interpret as neurosecretory axons.

We have found possible peritrophic membrane associated with the cardial epithelium and with the anterior midgut epithelium (Fig. 9). This membrane is not always present, but appears to occur both in mosquitoes fed only sugar and in blood-fed mosquitoes. Much more work needs to be done on this.

b. IT time series & IF route

Figure 10 indicates that antigen distribution (infection) follows a distinct temporal sequence, the IF cells becoming infected first followed by the ACE, and then the CE cells. In all cases, there was spatial continuity of antigen, i.e. antigen-positive staining in the IF was continuous with staining of the ACE cells which in turn was continuous with staining of the CE.

c. Additional Testing of the Foregut-Midgut Dissemination Hypothesis

Orally infected specimens (fed on a viremic hamster or viremia blood from a gauze pad) with non-disseminated

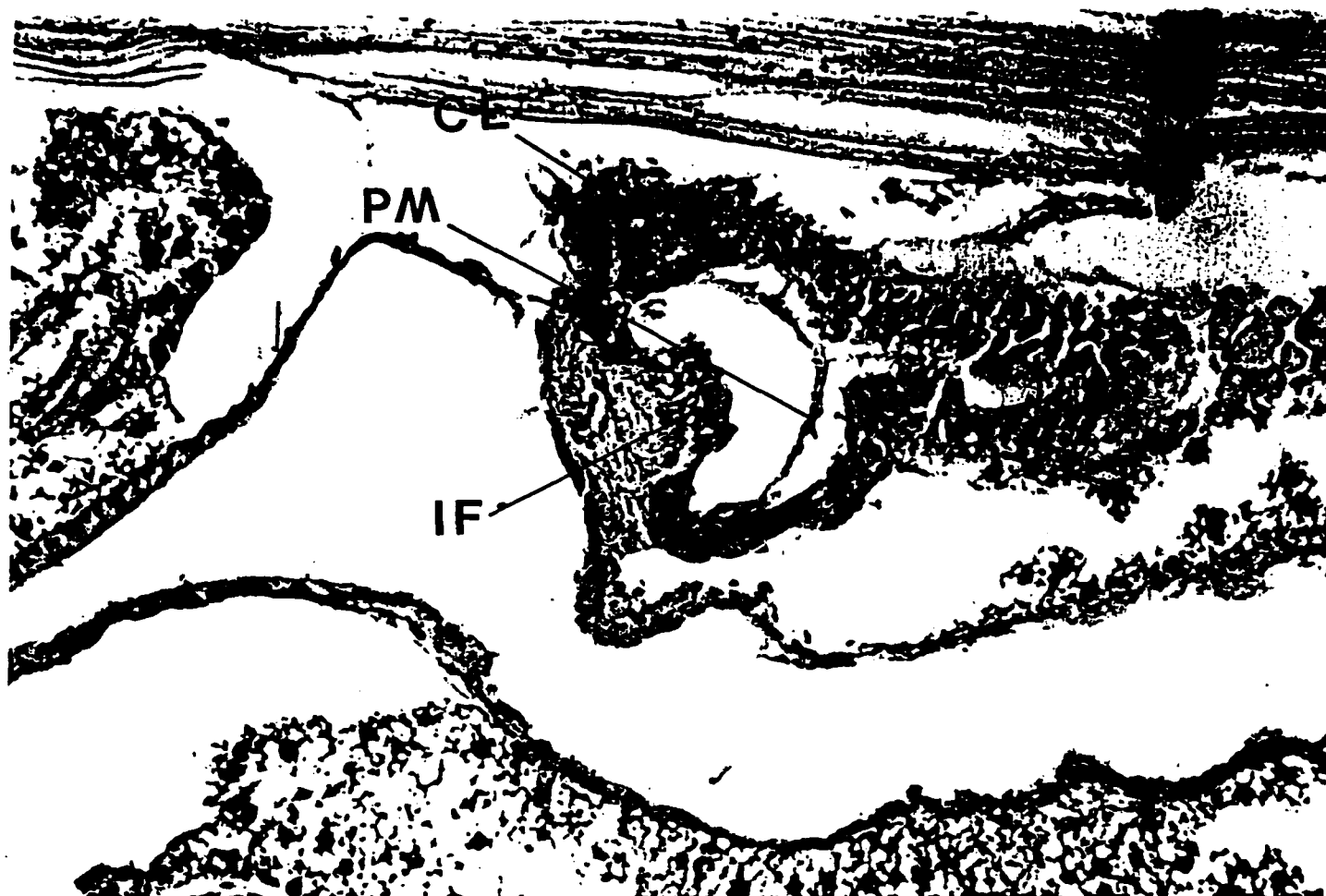
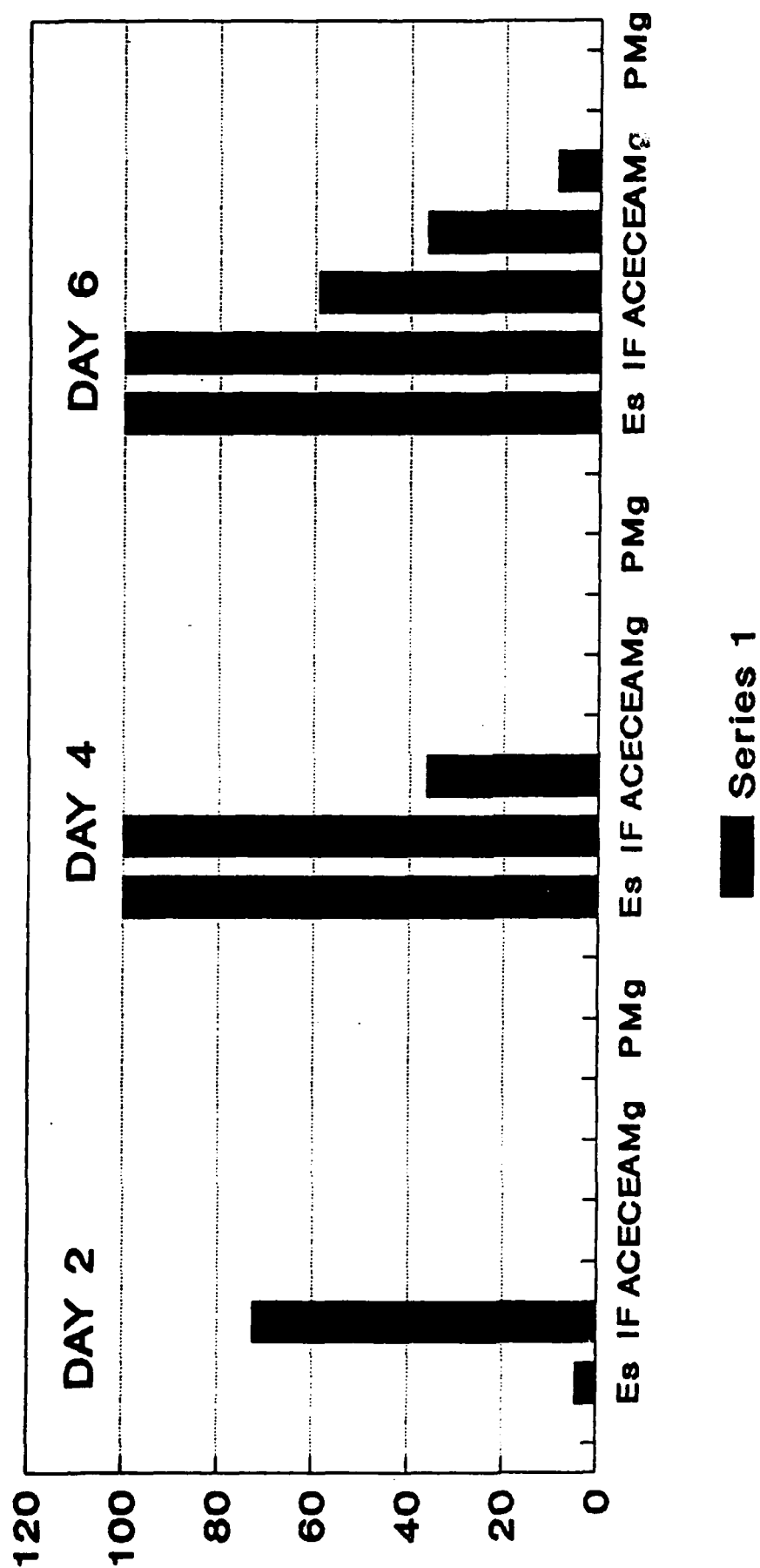


Fig. 9. Light photomicrograph of sagittal section of proventriculus of *Culex pipiens*. CE, cardiac epithelium; Es, esophagous; IF, intussuscepted foregut; PM, putative peritrophic membrane; VD, ventral diverticulum.

FIG.10 RVFV ANTIGEN DISTRIBUTION IN GUT
TIME POST-IT INFECTION (n=22/day)



Es=esophagus; IF=intuss. foregut;
 ACE=ant. card.; CE=cardial epith.;
 AMg=ant. midgut; PMg=post. midgut

Infections were prepared for electron microscopic examination. Out of 19 specimens examined, we found putative virions in the IF of 15.

d. Locations of Virions in the Proventriculus.

Within the proventriculus, we have seen putative RVF virions (Fig. 11) in the "spongy" basal lamina (Fig. 12 & 13, SBL), in the "endocuticle" of the foregut intima (Fig. 14), in tracheal/tracheolar cells (Fig. 15), in nerve fibers (Fig. 16), in the basal labyrinth of cardiac epithelial cells (Fig. 17), and in foregut epithelial cells (Fig. 18). In addition, we have seen budding of putative RVF virions directly into the basal labyrinth (Fig. 17) and directly into the "endocuticle" (Fig. 14). We have also seen apparent assembly of RVF virions in association with smooth membranes within foregut epithelial cells (Fig. 19). These observations are consistent with the findings of Anderson & Smith (1987) in rat hepatocytes.

e. General Discussion of RVF Virus in the Proventriculus

Our reasons for interest in the proventriculus (cardia) are as follows: (1) recent evidence suggests that Rift Valley fever virus may disseminate from the gut into the foregut and hemocoel via cells at the foregut-midgut junction (Romoser et al., 1987; 199_); (2) the intussuscepted foregut, part of the proventriculus is typically the first tissue infected following dissemination of RVF virus from the midgut; following intrathoracic injection of RVF virus and a short incubation period, the intussuscepted foregut infection rate is 100%, making this an ideal tissue in which to study viral morphogenesis, i.e. one can always find virus in it; (3) given its location between the foregut and midgut, the proventriculus is involved in the passage of both blood and nectar meals into the midgut; and (4) given the complexity of the proventriculus in mosquitoes, other diptera, and other insects, this organ is of interest from a systematic and evolutionary point-of-view.

The results described above constitute additional evidence of cells at the midgut-foregut junction provide a route of egress of RVF virus from the midgut.

The spatial continuity of and temporal sequence of infection (antigen distribution) in IT-infected mosquitoes are consistent with the spreads of virus from cell to cell in the proventriculus. Further, the presence of putative

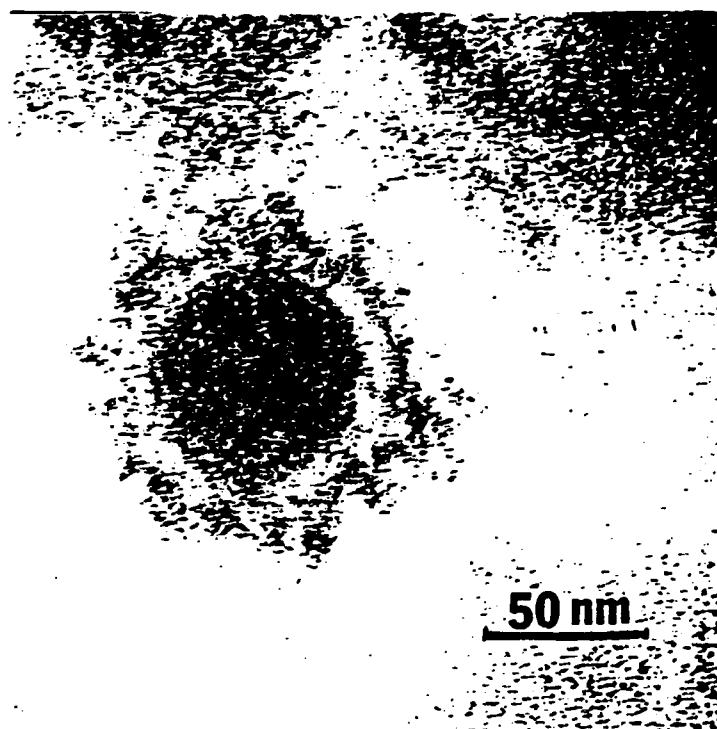


Fig. 11. Electron micrograph of putative Rift Valley fever virion in Culex pipiens.

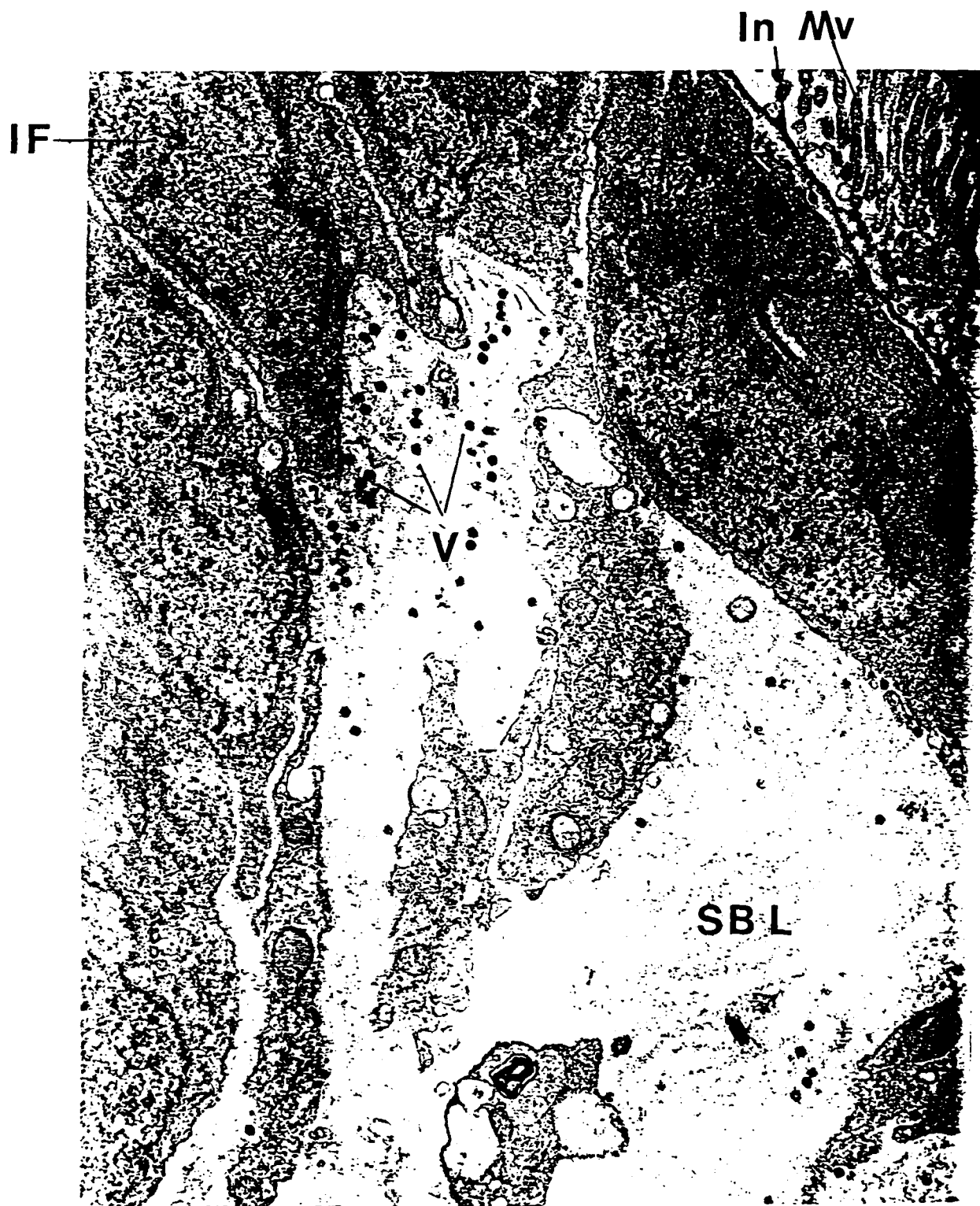


Fig. 12. Electron micrograph of putative RVF virions (V) in the "spongy" basal lamina (SBL) of the proventriculus of Culex pipiens; IF, intussuscepted foregut; In, foregut intima; Mv, microvilli.

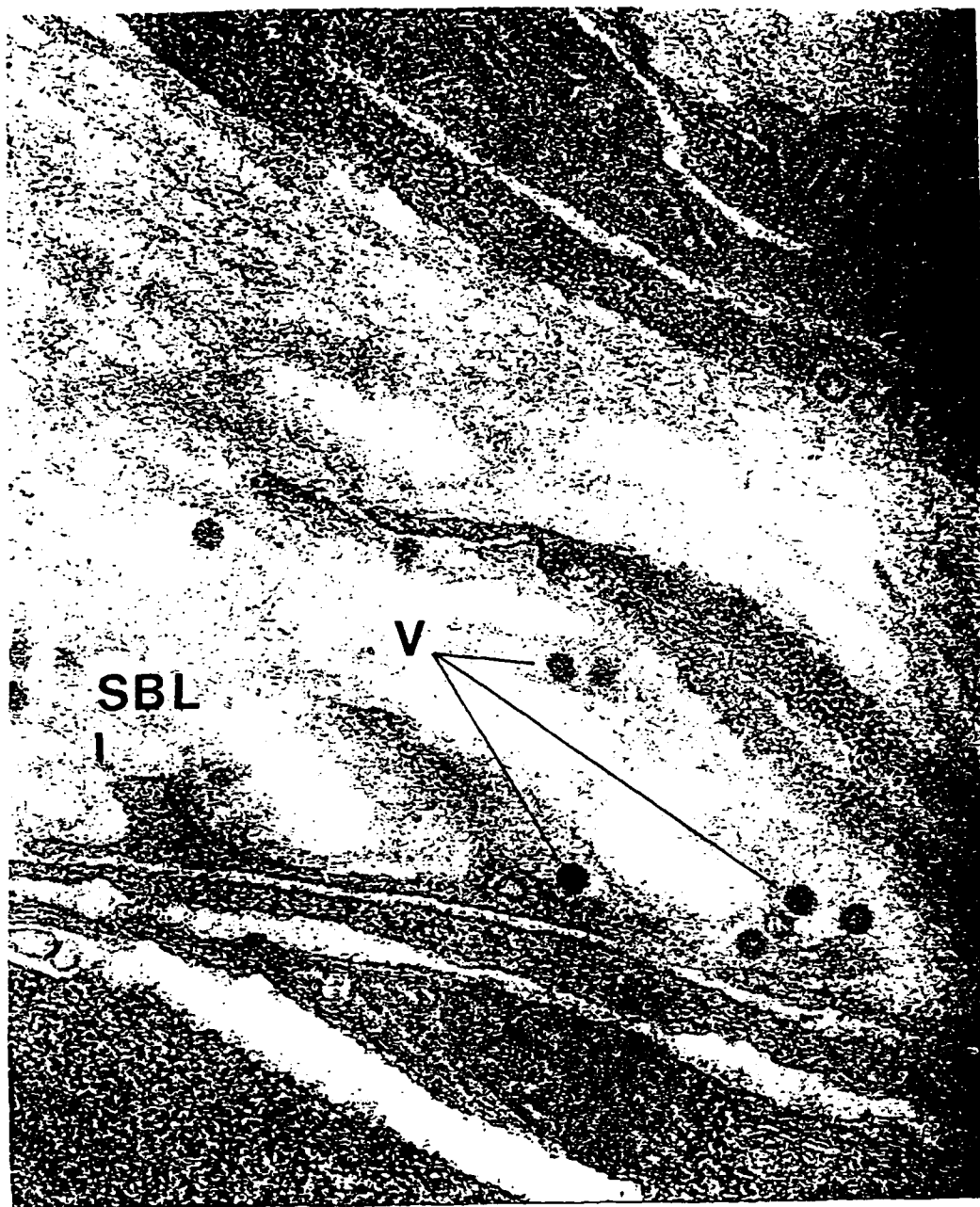


Fig. 13. Electron micrograph of putative RVF virions (V) in the "spongy" basal lamina (SBL) of the proventriculus of Culex pipiens.

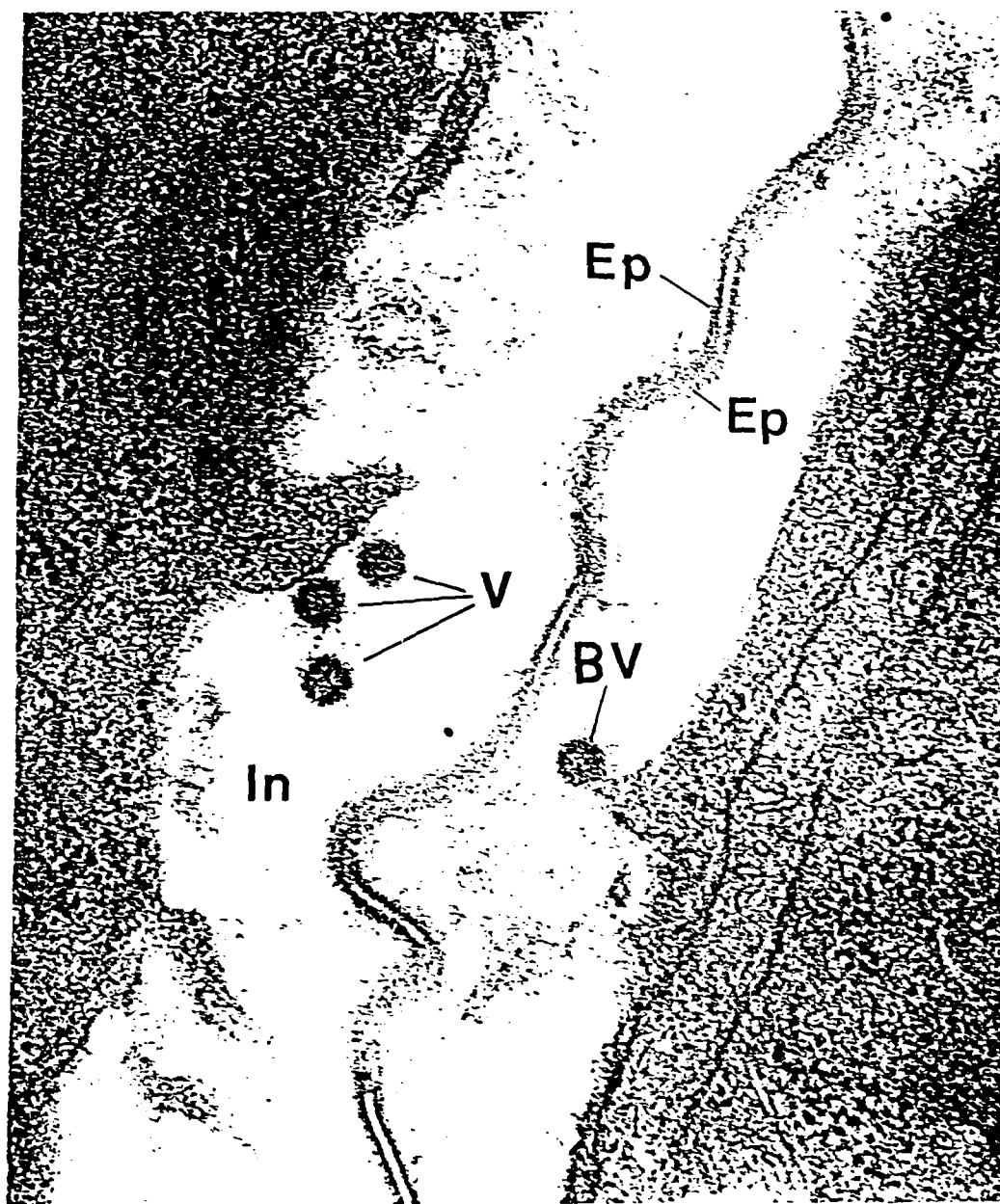


Fig. 14. Electron micrograph of putative PVF virions (V) in the "endocuticle" of the foregut intima (In). Note the apparent budding virion (BV). The "epicuticle" (Ep) appears double because two layers of intima are appressed to one another in this section.



Fig. 15. Electron micrograph of putative RVF virions (V) in the cytoplasm of a tracheal cell within the proventriculus of *Culex pipiens*. Cy, cytoplasm of tracheal cell; Tr, lumen of trachea.

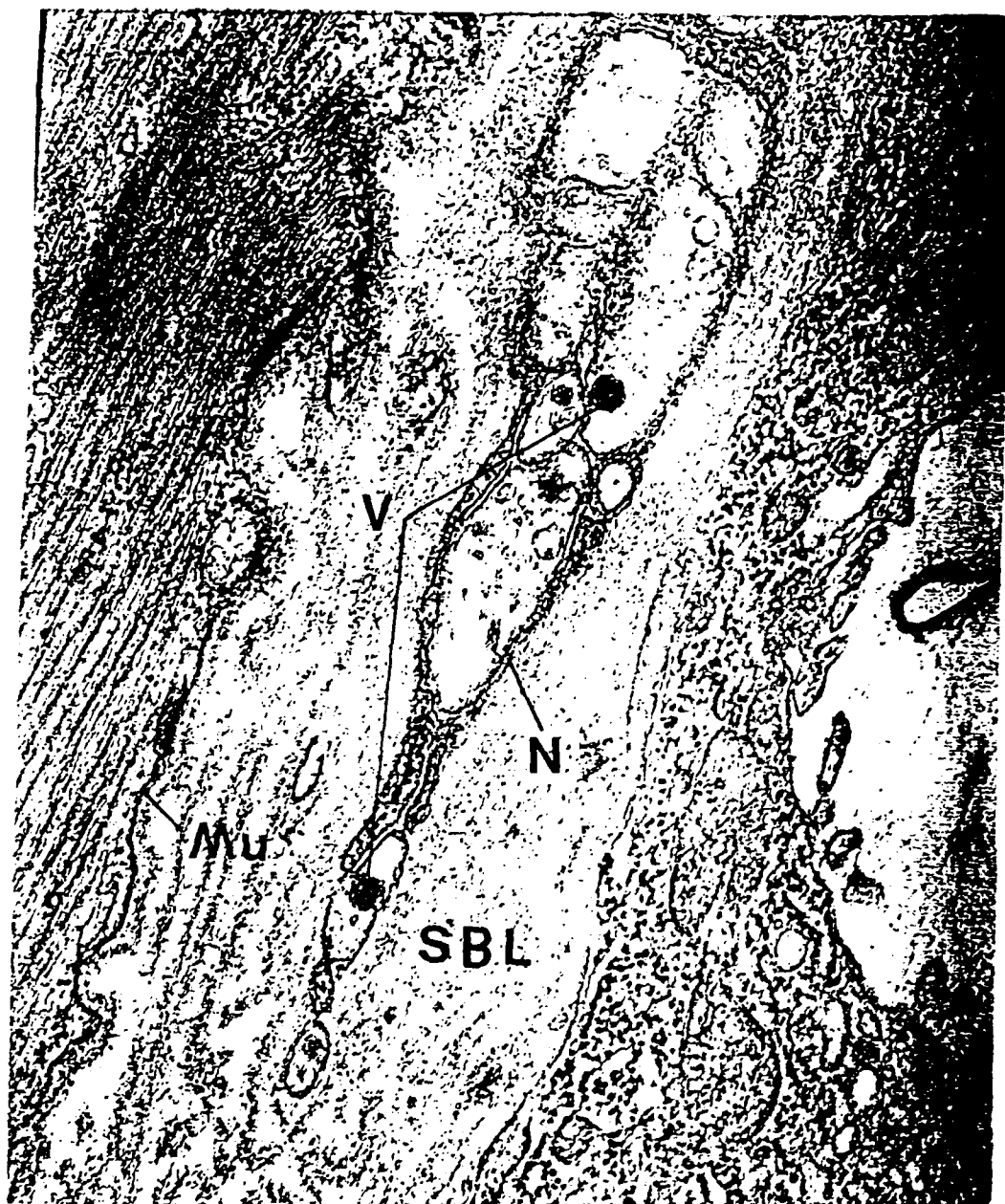


Fig. 16. Electron micrograph of section of nerve fiber (N) in the proventriculus of Culex pipiens. Mu, muscle; SBL, "spongy" basal lamina; V, putative RVF virions.

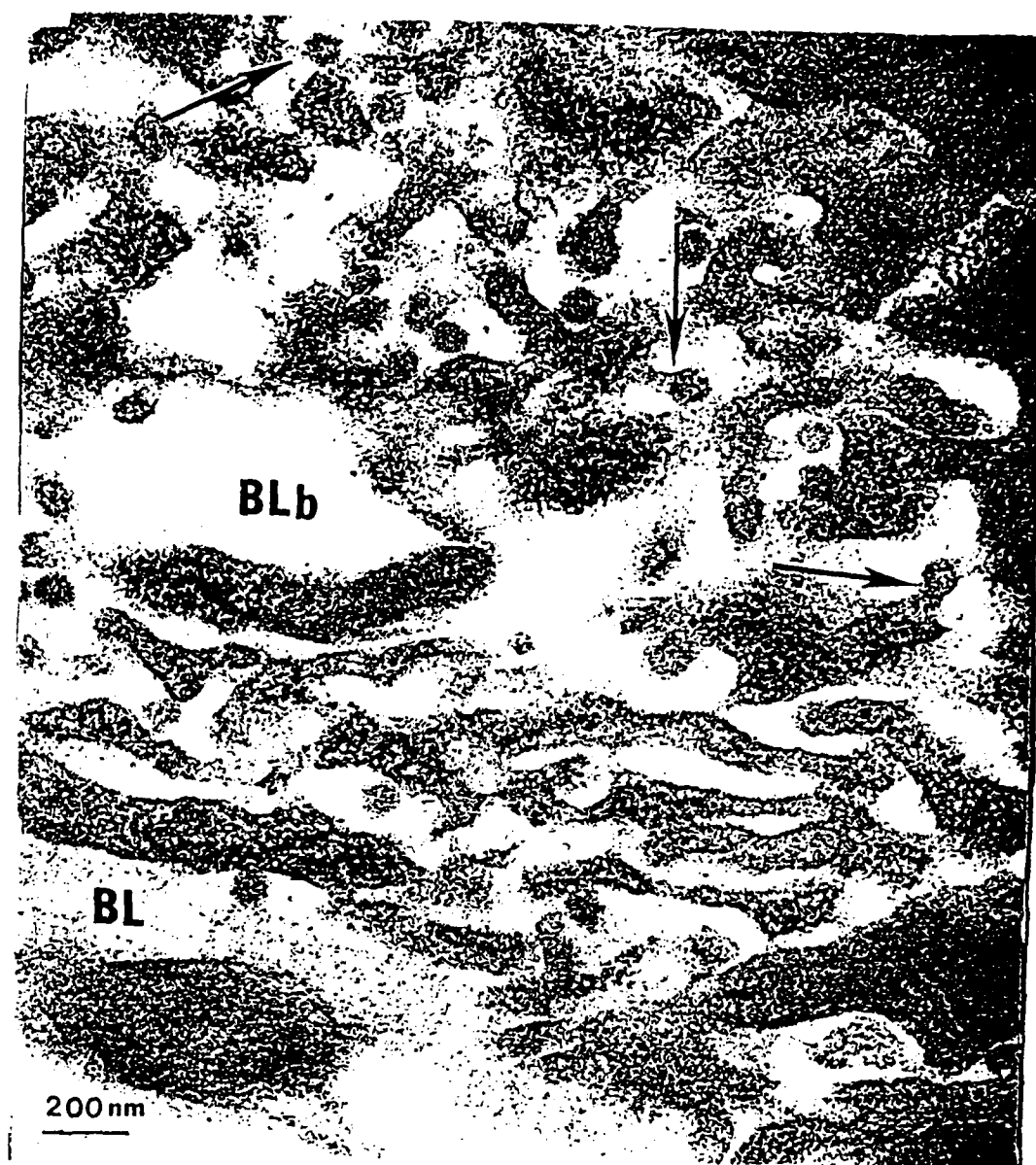


Fig. 17. Electron micrograph of basal labyrinth (BLb) in cardiac epithelial cell of Culex pipiens showing budding of putative RVF virions (indicated by arrows). BL, basal lamina of midgut.

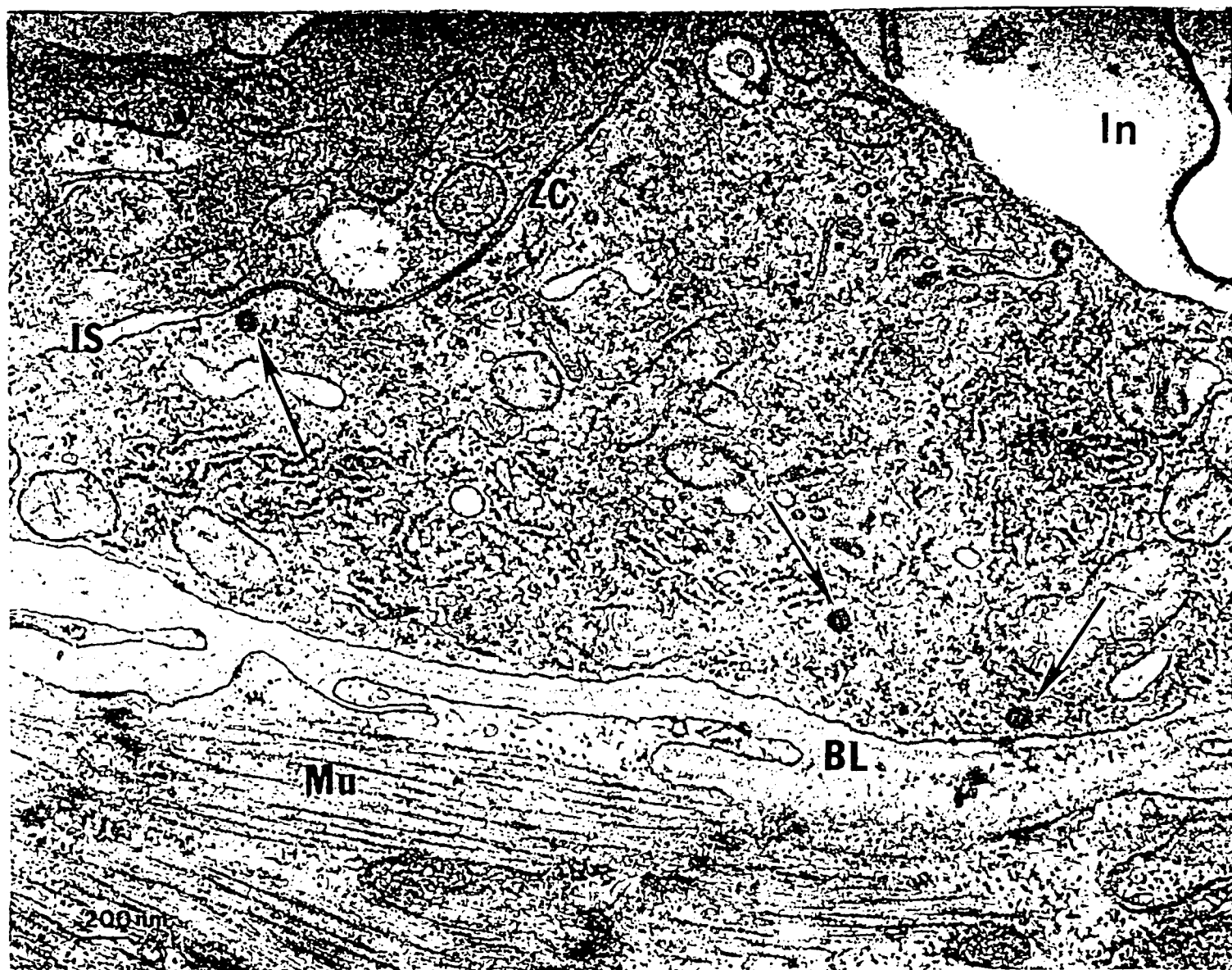


Fig. 18. Electron micrograph of foregut epithelium of *Culex pipiens* showing putative RVF virus (indicated by arrows). BL, basal lamina of foregut epithelium; In, foregut intima; IS, intercellular space; Mu, muscle; ZC, zonula continua.

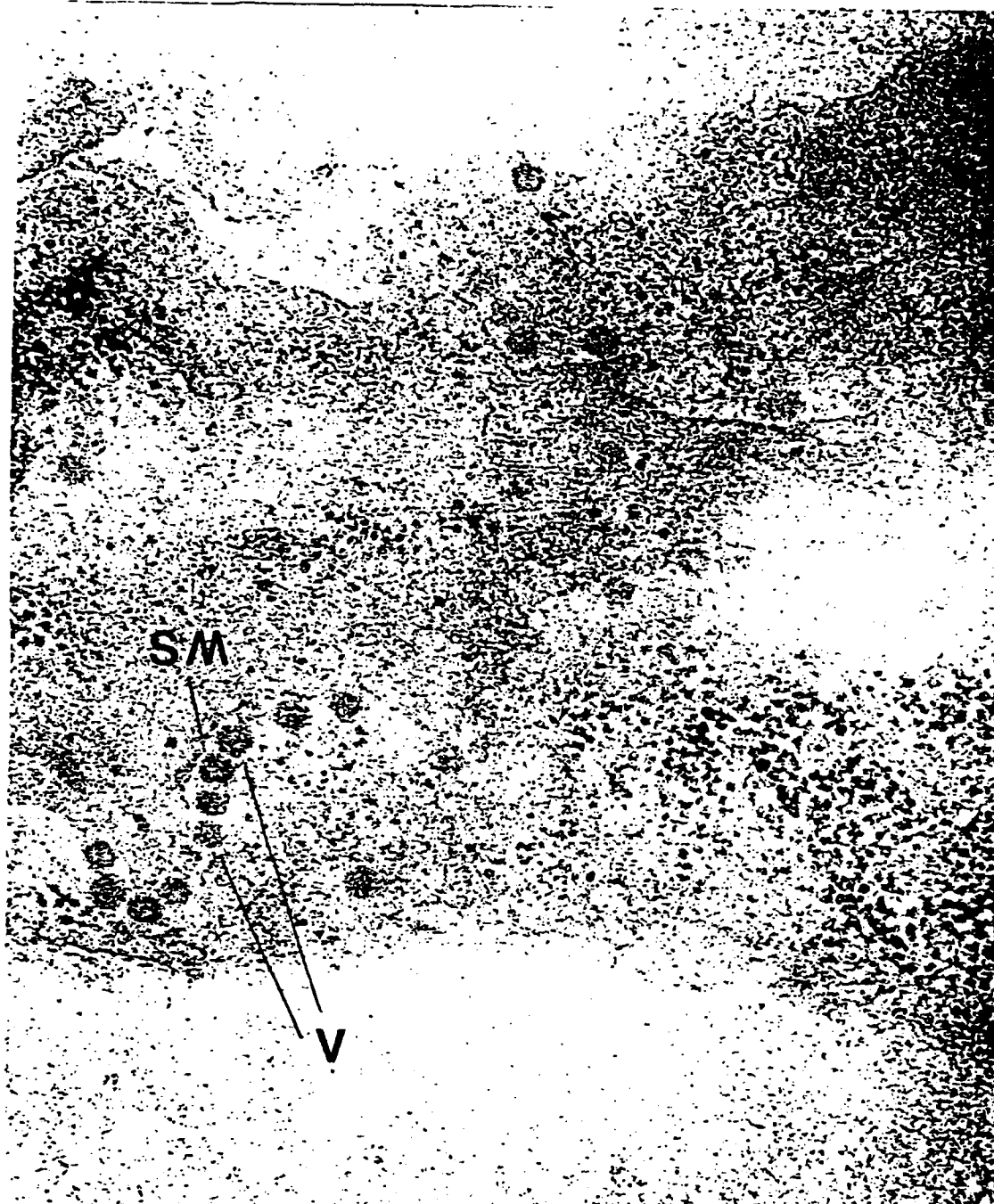


Fig. 19. Electron micrograph of putative RVF virions (V) being assembled in association with smooth membrane (SM) in an intussuscepted foregut epithelial cell of the proventriculus of Culex pipiens.

RVF virions in the IF of orally infected specimens with non-disseminated infections provides further evidence of infection of the ACE cells from the midgut lumen and the dissemination of virus within these tissues. Infection probably occurs most commonly on the microvilli of the ACE cells followed by infection of the IF cells by cell-to-cell spread of virus. Once virus has reached the IF cells it seems likely that it can be shed into the "spongy" basal lamina and work its way into the hemocoel or continue into the foregut epithelium anterior to the proventriculus by cell to cell spread. The nature of intercellular boundaries in the foregut epithelium would appear to be ideal for cell to cell spread of virus since between each cell the zonula continua (ZC) do not reach the cell bases (Fig. 18). Thus spaces occur between the basal regions of adjacent cells and viruses shed from one cell would have direct and immediate access to plasma membrane of an adjacent cell. Our ultrastructural studies also indicate that virus can infect nerve fibers and tracheal/tracheolar cells in the proventriculus. Virus may thus also move via these cells. In addition, it is possible that virus can infect adjacent cells by entering the "endocuticle" of the foregut intima, since it is clearly able to but into the substance which composes this layer.

2. Anopheles Salivary Gland Barrier

a. The results of our analysis of the immunocytochemically stained slides are shown in Tables 1-3.

Table 1

Viral transmission and antigen presence and distribution in Anopheles stephensi as a function of developmental stage at infection.*

Developmental Stage Infected	Trans- mission	Antigen Detected	Antigen in IF	Salivary Glands
larval	90 (9/10)	100 (10/10)	100 (10/10)	80 (8/10)
pupal	38.9 (7/18)	100 (18/18)	55.6 (10/18)	61.1 (11/18)
adult	0 (0/20)	95 (19/20)	15 (3/20)	5 (1/20)
uninfected controls	0 (0/10)	10 (1/10)	0 (0/10)	0 (0/10)

* Upper number = percent; numbers in parentheses = number transmitting or number RVF viral antigen positive / number examined.

Table 2

Distribution of RVF viral antigen in the salivary glands of Anopheles stephensi as a function of developmental stage at infection.*

Developmental Stage Infected	Antigen in Salivary Glands	
	Proximal lobes	Distal lobes
larval	80(8/10)	30(3/10)
pupal	50(9/18)	16.7(3/18)
adult	5(1/20)	0(0/20)
uninfected controls	0(0/10)	0(0/10)

* Percent (number RVF viral antigen positive/number examined).

Table 3

Distribution of RVF viral antigen in the Intussuscepted foregut (IF) and salivary gland regions in virus-transmitting specimens of Anopheles stephensi as a function of developmental stage at infection.*

Developmental Stage Infected	IF	Salivary Gland Regions			
		Either**	Distal Only	Proximal Only	Both**
larval	100 (9/9)	88.9 (8/9)	0 (0/9)	44.4 (4/9)	33.3 (3/9)
pupal	85.7 (6/7)	85.7 (6/7)	28.6 (2/7)	42.9 (3/7)	14.3 (1/7)
all transmitters	93.8 (15/16)	87.5 (14/16)	12.5 (2/16)	43.8 (7/16)	25 (4/16)

* Upper number = percent; lower numbers in parentheses = number RVF viral antigen positive/number examined.

** Either the proximal or distal; both the proximal and distal.

b. Conclusions & Discussion

(1) The ABC technique proved to be quite reliable in determining whether or not a specimen was infected with virus. Antigen was detected in 97.9% (47/48) of the IT-infected specimens and the ABC technique produced negative results in 90% (9/10) of the uninfected specimens. Relative to the ABC technique, there was one false negative and one false positive. In addition, there was one false negative for the plaque assay technique on Vero cells, i.e. the specimen contained RVF viral antigen (based on the ABC technique) and transmitted virus to a hamster.

(2) As Turell (1988) has shown, the more advanced the developmental stage (larva, pupa, adult) when IT-infected with RVF virus, the lower the rate of transmission by the adult stage (Table 1). That is, among adults produced from IT-infected larvae, 90% transmitted virus to hamsters, among adults produced from IT-infected pupae, 38.9 % transmitted virus, and among adults which were IT-infected as adults and incubated a period of time equivalent to those infected as larvae and pupae, 0% transmitted.

(3) In association with decreased transmission as a function of infection in more advanced developmental stages, the frequency of salivary gland infection also decreases (Tables 1 & 2).

(4) Since no mosquitoes infected as adults transmitted virus and only 1 in 20 had detectable antigen in the salivary glands (Tables 1 & 2), it appears that there is a salivary gland barrier operating.

(5) Since transmission by the adult decreases the more advanced the developmental stage and since some adults infected as pupae do not transmit, it appears that the salivary gland barrier develops some time during the pupal stage (Table 1).

(6) Five percent (1/20) of the specimens IT-infected as adults contained antigen in their salivary glands, but did not transmit virus (Table 1). However, it is possible that the salivary gland-infected adult would have eventually become competent to transmit virus. Further, the fact that the salivary glands became infected in one adult may indicate that, given enough time, other IT-infected adults may develop salivary gland infections. It seems likely that the salivary gland infection barrier that appears to be indicated by our data is time dependent.

(7) Like the salivary glands, the IF appears to become less susceptible to infection when infection occurs during the adult stage (Table 1).

(8) One might argue that virus may replicate at different rates depending on the timing of infection relative to developmental stage. However, Turell (1988), studying Anopheles albimanus, found, that when incubation times were equivalent, similar viral titers were produced regardless of the timing of infection.

(9) The occurrence of transmitters in which only the distal regions or only the proximal regions of the salivary gland lobes are infected is evidence that transmission can occur via either of these regions (Table 3). However, among all transmitters, the proximal region showed the highest frequency of infection (Table 2).

(10) Since 87.5% (14/16) of the transmitting mosquitoes had antigen in at least one region of the salivary glands (Table 3), the presence of antigen in the salivary glands would appear to be a good, but not perfect predictor of the ability to transmit. Ironically, infection of the intussuscepted foregut (IF) appears to be an even better predictor of the ability of an individual to transmit virus given that 93.8% (15/16) of the specimens which transmitted contained RVF viral antigen in cells of this structure (Table 1).

B. cDNA Studies

A preliminary in situ hybridization experiment using Culex pipiens intrathoracically infected with RVF virus showed hybridization of RVF virus nucleic acid with cDNA probe as indicated by a purplish-blue color. Specifically stained tissues included those tissues in Cx. pipiens that exhibited specific staining using the avidin-biotin-peroxidase complex (ABC) immunocytochemical procedure for detection of RVF virus antigen (Faran et al., 1986). The in situ hybridization technique, however, does not show the non-specific staining of the ABC technique in the Malpighian tubules and pericardial cells, rather specific staining of these tissues in addition to the tracheal cells. Furthermore, melanized cuticle is not the same color as positively stained tissues as is the case in the ABC procedure.

The nuances of the in situ hybridization procedure are still being worked out. The most efficient hybridization of the probe and least background occurs with sections fixed in

4.0% paraformaldehyde or an ultrapure formaldehyde solution, and 4.0% paraformaldehyde will be used on future specimens. In October, 1988, Aedes mcintoshi and Ae. circumluteolus were orally exposed to RVF virus and later fixed in 10% neutral buffered formalin. Several serial sections of these mosquitoes showed little staining. Pretreatment of sections by partial proteolytic digestion by proteinase K or pronase has improved hybridization of the probe. However, this treatment has also produced some non-specific staining, particularly in the thoracic musculature. I hope to compare these mosquitoes with those to be fixed in 4.0% paraformaldehyde. As time permits, preference will be given to examining Ae. mcintoshi and Ae. circumluteolus as they become available from Kenya.

C. Search for the Receptor

1. Results--Approach #1

a. The two microtiter assays described using a variety of different targets all demonstrated that RVF virus prepared in the form of a formalin-killed vaccine has a specific binding capacity to components of these targets. The biotinylated viral protein was found to have greater sensitivity and higher specificity than the use of monoclonal antibodies to detect viral binding extracts.

b. It must be noted that the binding demonstrated here does not necessarily constitute cell surface receptors and can only be a conjecture which, of course, is tempting.

c. We have corroborated our microtiter assays by visualizing binding using fluorescence.

2. Results--Approach #2

a. The results of the two plaque inhibition assay experiments are shown in Tables 4 and 5.

b. Strong reduction in the number of plaques (i.e. 54% in both replicates 1 & 2) was caused by the mouse designated NO E when the serum and Vero cells were incubated for one hour prior to the addition of virus. No such reduction was evident when virus & serum were mixed and added to the cells together. These results suggest that antibodies to mosquito cell surface molecules blocked RVF virus attachment. The results are especially interesting in view of the fact that the mouse antibodies are directed against mosquito cells and the inhibition of plaque formation took place on vertebrate cells, i.e. Vero cells. It is also significant that the mouse sera were diluted 1:50. We would expect that lower dilutions would effect even greater inhibition of plaque formation and plan to do dose response studies with monoclonal antibodies derived from the "active" mouse's serum. We also plan to test for plaque inhibition using Aedes albopictus C6/36 cells instead of Vero cells.

Table 4

Influence of mouse (Balb/c) serum containing antibodies directed against mosquito cell surface molecules (Aedes albopictus C6/36 cells) on plaque formation on Vero cell monolayers infected with RVF virus (Replicate 1).

Treatment	Plaques/ Well	\bar{X} Plaques/ Well	% Plaque Reduction*
Cells Incubated with Sera:			
MS	136, 130	133	10
RE	117, 130	124	16
LE	134, 122	128	14
NO E	71, 64	68	54
Control 1, Virus Only			
	150, 146	148	-
Control 2, Virus + Sera:			
MS	137, 141	139	6
RE	146, 143	145	2
LE	144, 149	147	1
NO E	133, 115	124	16

* % Plaque reduction as compared with the number of plaques in the "virus only" wells. Mean values and percent values rounded to nearest whole number.

Table 5

Influence of mouse (Balb/c) serum containing antibodies directed against mosquito cell surface molecules (Aedes albopictus C6/36 cells) on plaque formation on Vero cell monolayers infected with RVF virus (Replicate 2).

Treatment	Plaques/ Well	\bar{X} Plaques/ Well	% Plaque Reduction*
Cells Incubated with Sera:			
MS	139, 132	136	6
RE	124, 136	130	10
LE	132, 127	130	10
NO E	66, 68	67	54
Control 1, Virus Only			
	143, 147	145	-
Control 2, Virus + Sera:			
MS	133, 138	136	6
RE	140, 135	138	5
LE	136, 135	136	6
NO E	130, 127	129	11

* % Plaque reduction as compared with the number of plaques in the "virus only" wells. Mean values and percent values rounded to nearest whole number.

D. Preliminary &/or Equivocal Results

1. Application of Immunogold Techniques for Use in Ultrastructural Studies of Mosquito Cells Infected with RVF Virus:

Our results as compared to attempts using colloidal gold-protein A have shown promise, but we have not yet achieved consistent, unequivocal labelling of RVF virions in situ.

IV. Studies in Progress

A. Detailed immunocytochemical studies of various African species

We have been collecting data for some time regarding the fate of RVF virus in orally-infected and/or IT-infected African mosquito species and are currently completing data collection. We will report the results of these studies in our next annual report.

B. Further evaluation of Salivary Gland Barrier in Anopheles

We are planning to replicate the experiment described in this report. We are also preparing histological and ultrastructural material in order to study the development of the salivary glands and will try to find correlations between a developmental event or events and the beginning of operation of the "salivary gland infection barrier". If we are successful in developing monoclonal antibodies to the mosquito cell surface receptor for RVF virus, we can use these antibodies to evaluate the salivary gland barrier with regard to the presence, absence, density, and/or blockage of viral receptor molecules on the salivary glands.

C. Further histological and ultrastructural studies of the proventriculus and of the putative peritrophic membrane which appears to form in the proventriculus and anterior midgut.

D. Application of the cDNA for In situ Studies

The in situ hybridization assay is being worked out on Culex pipiens, an epizootic vector, intrathoracically inoculated with RVF virus. Aedes

mcintoshi eggs recently obtained from an enzootic site in Kenya were reared to adults and intrathoracically inoculated with RVF virus, and held for 7 days at 26°C before fixation. Additional Ae. mcintoshi females will be orally exposed to RVF virus and mosquitoes will be fixed at various time intervals post-feeding to study viral morphogenesis and dissemination.

E. Search for the Receptor

1. Continuation of Fractionation of Mosquito Cell Membrane Extracts and Probing with Virus Preparations

2. In order to produce monoclonal antibodies, spleenocytes will be fused to Sp20 myeloma cells and cultured in the presence of HAT. Surviving hybridoma supernatants will be screened by an ELISA using 3 different albobictus cell preparations: (1) glutaraldehyde (0.25%) fixed cells; (2) methanol-fixed cells; and (3) solubilized membrane preparations. Hybridoma lines secreting positive antibodies will be cloned and then screened for the ability to inhibit plaque formation in both Vero cells and Aedes albopictus C6/36 cells.

Hybridoma lines secreting antibodies of interest will be expanded and used for ascites production. Monoclonal antibodies will be purified, cleaved into Fab fragments and subsequently re-assayed for plaque reduction. Antibodies will also be used in affinity columns and Western blots to determine the antigen to which they bind.

F. To continue development of the application of in situ immunogold-labelling in ultrathin sections in order to (1) provide validation that the particles we are interpreting as RVF virions are, in fact RVF virions, and (2) facilitate detailed studies of RVF virus morphogenesis.

G. Work with Ticks:

In collaboration with Cpt. Scott Gordon at USAMRIID, we are studying the presence of RVF virus and Congo-Crimean Hemorrhagic fever virus in ticks using electron microscopy. In addition, we are working on application of the ABC immunocytochemical technique and other similar techniques to study viral antigen distribution in various tick species.

H. In order to see if the ABC technique is applicable to other arboviruses using polyclonal antisera, we have begun limited study of Ockelbo virus in Culex pipiens in

collaboration with Dr. M. Turell, USAMRIID and Mr. Jan Lundstrom, Sweden.

I. We are continuing to look for RVF virus in ultrathin sections of various mosquito tissues in order to gain more insight into morphogenesis of this virus in mosquitoes.

J. We are studying the effects of various extrinsic factors (temperature, nutrition, interrupted-feeding, etc.) on the infection process and dissemination of RVF virus in Culex pipiens.

K. We have and are continuing to collect light and electron photomicrographs for our monograph on mosquito histology and ultrastructure.

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VI. FINAL SUMMARY OF PROJECT

A. Goals, Methods, Subprojects, and Major Findings

During the past three years, we have been engaged in a multimethod study of Rift Valley fever (RVF) virus in mosquitoes, including a species thought to have been involved in a major epidemic/epizootic, Culex pipiens; species thought to be involved in the interepidemic/interepizootic maintenance of RVF virus, Aedes mcintoshi and others; and Anopheles spp. in which a putative salivary gland infection barrier has been identified.

Our overall goal has been to contribute to the body of knowledge on the epidemiology of Rift Valley fever by studying the dissemination, pathogenesis and morphogenesis of the RVF virus in vector competent and incompetent mosquitoes and by identifying, isolating and characterizing the mosquito cell surface receptor molecule to which RVF virus binds. Through achieving the above goals, it has been, and remains, our hope to shed light on various intrinsic factors which influence vector competence.

Specific questions we have been addressing (pertinent both intraspecifically and interspecifically) include: (1) Why do some mosquitoes fail to develop midgut infections? ("midgut infection barrier"). (2) Why do some mosquitoes develop midgut infections, but fail to develop disseminated (systemic) infections? ("midgut escape barrier"). (3) Why do some mosquitoes with disseminated infections fail to transmit virus to their offspring or to vertebrate hosts? ("salivary gland barriers" and "ovarian barriers"). (4) What is the mechanism associated with "early dissemination" & "rapid transmission"? (5) What are the effects of temperature, viral dose, interrupted feeding, nutritional state, heredity, etc. on the operation of the various barriers? (6) Is the operation of a given "barrier" due to the absence of an appropriate receptor molecule on the surface of host cells? ...due to a low density of receptor molecules? ...due to something preventing contact between virus and cell surface receptor molecules such as basal lamina?

The methods we have applied include viral titration by plaque assay, light & transmission electron microscopy, light & electron immunocytochemistry, the use of a cDNA probe, electrophoresis, Western blotting, column chromatography, ELISA, various other biochemical techniques, and monoclonal antibody technology.

Specific studies have included: (1) An immunocytochemical study of RVF virus infection and dissemination in Cx. pipiens. (2) An immunocytochemical study of field-collected Aedes mcintoshi. (3) an immunocytochemical study of RVF virus in the male and female reproductive systems of Ae. mcintoshi. (4) an immunocytochemical survey of field-collected Ae. mcintoshi for potential natural infection with RVF virus. (5) Studies of the ultrastructure of the proventriculus of Culex pipiens with regard to possible dissemination of RVF virus via cells at the foregut-midgut junction and with regard to RVF virus morphogenesis. (6) Immunocytochemical studies of the possible salivary gland barrier in Anopheles spp. (7) Studies to develop the application of a cDNA probe for the in situ localization of RVF virus in paraffin sections of mosquitoes. (8) Studies to develop the application of an immunogold technique for the in situ labelling of RVF virions in ultrathin sections. (9) Studies with regard to identifying the mosquito cell surface receptor molecule for RVF virus.

Major results and conclusions from the above studies include: (1) Early dissemination and possible early or rapid transmission can occur in Culex pipiens. (2) Evidence from several standpoints supports the idea of virus escape from the gut lumen in the region of the foregut-midgut junction. (3) the midgut basal lamina may act as an escape barrier or at least retard viral passage. (4) RVF virus is capable of infecting most tissues in the mosquito hemocoel and probably exerts its pathological effects both by a general depletion of energy stores and by interference with regulatory processes. (5) both the fat body and foregut may be important amplifying tissues. (6) The patterns of midgut infection, escape of virus from the midgut, and distribution of virus after entering the hemocoel in Ae. mcintoshi are similar to those we found earlier in Cx. pipiens and support or are consistent with the conclusions derived from studying the later species. (7) We have found immunocytochemical evidence that the follicular epithelia and eggs of Ae. mcintoshi can become infected by virus in the hemolymph. (8) Further, in male Ae. mcintoshi, the testicular sheath cells contained antigen in over one-third of the 13 male mosquitoes examined. It is therefore possible that spermatozoa could become infected, but we observed no evidence of this. (9) Out of 1771 Ae. mcintoshi collected from dambos in Kenya, we found one specimen that appeared to be RVF viral antigen-positive, but none of the same 1771 specimens were RVF virus-positive based on plaque assay. As a result of this work, we developed a way to examine large numbers of mosquitoes immunocytochemically in a relatively efficient manner. (10)

Immunocytochemical evidence suggests strongly the existence of a salivary gland infection barrier in Anopheles spp. and it is possible that this barrier is time-dependent. (11) The development of the application of a cDNA probe for the in situ localization of RVF viral genome in paraffin sections has been successful and will be used, for example, in future studies of the female reproductive system in infected female Aedes mcintoshi. (12) We have shown that some portion of the both formalin-killed RVF vaccine virus and of the HA preparation bind to molecules on the surface of mosquito cells. (13) We have shown that a polyclonal serum to a cell membrane extract prepared from Aedes albopictus cells causes a significant reduction in the number of RVF viral plaques formed in Vero cell monolayers.

B. Publications

Romoser, W.S., M.E. Faran, & C.L. Bailey. 1987. A Newly recognized route of arbovirus dissemination from the mosquito midgut. *J. Med. Entomol.* 24:431-432.

Faran, M.E., M.J. Turell, W.S. Romoser, R.G. Routier, P.H. Gibbs, T.L. Cannon & C.L. Bailey. 1987. Reduced survival of adult Culex pipiens infected with Rift Valley fever virus. *Am. J. Trop. Med. Hyg.*, 37(2):403-409. (Note: this research was begun while Romoser was on sabbatical leave at USAMRIID, but most of the work on manuscript was done while supported by this grant at Ohio University.)_

Romoser, W.S. 1987. History of an Arboviral Infection in a Mosquito. *Proceedings of the Ohio Mosquito Control Assn.*, 17:51-57.

Faran, M.E., W.S. Romoser, R.G. Routier & C.L. Bailey. 1988. The distribution of Rift Valley fever virus in the mosquito Culex pipiens as revealed by viral titration of dissected organs and tissues. *Am. J. Trop. Med. Hyg.*, 39(2):206-213.

Several manuscripts for publication are in varying states of development.

C. Presentations

Is the basal lamina the mesenteron escape barrier to Rift Valley fever virus in mosquitoes? (with M.E. Faran & C.L. Bailey). Presented to the American Mosquito Control Association, April, 1986, New Orleans, Louisiana.

Mosquitoes & Arboviruses: Rift Valley Fever as a Model. Invited lecture presented to the Biology Dept., Heidelberg College, Tiffin, Ohio, Jan., 1987.

Dynamics of Rift Valley fever virus dissemination in mosquitoes as revealed by in situ localization techniques (part of a symposium entitled: Recent developments in mosquito vector biology worldwide). Presented to the American Mosquito Control Association, April, 1987, Seattle, Washington.

History of an Arboviral Infection in a Mosquito. Invited presentation. Ohio Mosquito Control Assn., Oct. 1987, Toledo, Ohio.

An immunocytochemical study of Rift Valley fever virus tissue tropisms in selected African mosquitoes (with K. Lerdthusnee, L.A. Patrican, K.J. Linthicum, D. Dohm & C. L. Bailey). Presented to The American Society of Tropical Medicine and Hygiene, Dec. 1987, Los Angeles, California.

Additional evidence for Rift Valley fever virus dissemination via cells at the foregut-midgut junction (with K. Lerdthusnee, K.J. Linthicum, D. Dohm & C.L. Bailey). Presented to the American Mosquito Association, Feb. 1988, Denver, Colorado.

Immunocytochemical Studies of Rift Valley Fever Virus in Selected African Mosquito Species. Invited lecture presented to the Dept. of Entomology, Michigan State University, Feb., 1988, E. Lansing, Michigan.

Immunocytochemical and ultrastructural studies of Rift Valley fever virus in selected African mosquito species (with K. Lerdthusnee, M.E. Faran, K.J. Linthicum, L.A. Patrican & C.L. Bailey). Poster presented to the XVIII International Congress of Entomology, July, 1988, Vancouver, B.C., Canada.

Evidence for the specific binding of Rift Valley fever virus to components of solubilized mosquito tissues and cultured cells (A.A. Mikhail presented; with M.G. Lozykowski). Poster presented to the American Society of Tropical Medicine and Hygiene, Dec. 1988, Washington, D.C.

Sites of Rift Valley fever virus infection in the proventriculus of adult Culex pipiens (with K. Lerdthusnee). Poster presented to the American Society of Tropical Medicine and Hygiene, Dec. 1988, Washington, D.C.

Histology and ultrastructure of the adult mosquito proventriculus (with K. Lerdthusnee). Presented to the American Mosquito Control Association, April, 1989, Boston, Massachusetts.

Detection of Rift Valley fever virus in mosquitoes by in situ hybridization (L.A. Patrican presented; with M.T. Vahey). Presented to the American Mosquito Control Association, April, 1989, Boston, Massachusetts.

Rift Valley fever as a model of mosquito-virus interaction. Invited lecture presented to the Dept. of Entomology, The Ohio State University, May, 1989, Columbus, Ohio.

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